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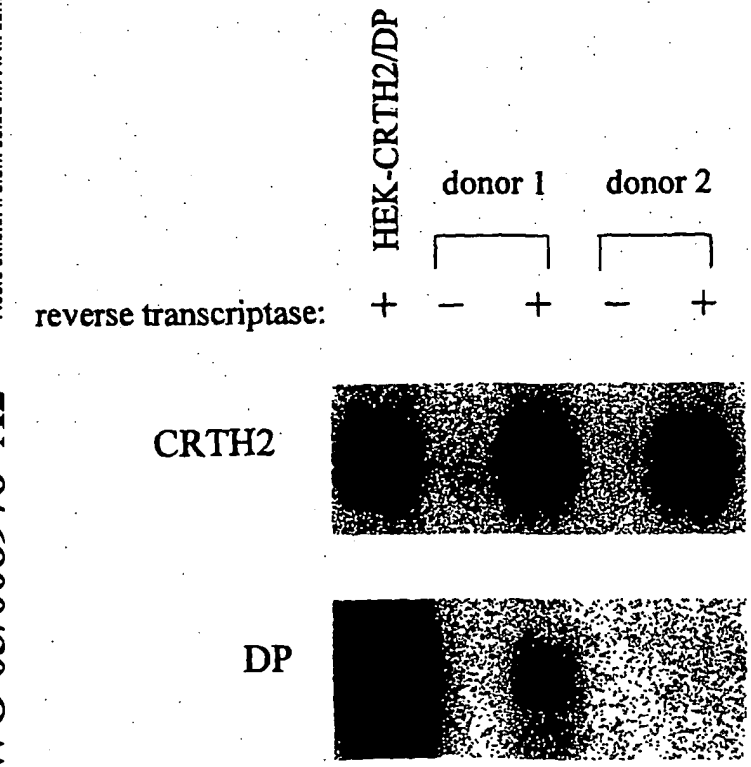
(54) Title: EOSINOPHIL PROSTAGLANDIN D_2 RECEPTOR ASSAYS



(57) Abstract: The present invention identifies different activities mediated by eosinophil PGD2 receptors and features methods measuring the ability of a compound to modulate such activities. Activities mediated by eosinophil PGD2 receptors include those associated with CRHT2 and those associated with the DP receptor. Activities identified herein as associated with eosinophil CRHT2 include a change in cell morphology, degranulation, and a specific chemokinetic effect. Activities identified herein as associated with the eosinophil DP receptor include resistance to apoptosis.

WO 03/008978 A2

TITLE OF THE INVENTION
EOSINOPHIL PROSTAGLANDIN D_2 RECEPTOR ASSAYS

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application claims priority to provisional application U.S. Serial No. 60/306,357, filed July 18, 2001, hereby incorporated by reference herein.

BACKGROUND OF THE INVENTION

The Background of the Invention and references cited in the present application are not admitted to be prior art to the claimed invention.

Prostanglandin D_2 (PGD_2) is a cyclooxygenase metabolite of arachidonic acid. (Narumiya, *et al., Physiological Reviews 79*:1193-1226, 1999.) PGD_2 has been implicated in playing a role in different physiological events such as sleep and allergic responses. (Boie, *et al., The Journal of Biological Chemistry, 270*:18910-18916, 1995, Narumiya, *et al., Physiological Reviews 79*:1193-1226, 1999, Matsuoka, *et al., Science 287*:2013-2017, 2000.)

Mast cells and TH2 cells are important immune cells involved in allergic responses. PGD_2 is released from mast and TH2 cells in response to an immunological challenge. (Roberts, *et al., N. Engl. J. Med. 303*:1400, 1980, Lewis, *et al., J. Immunol. 129:*1627, 1982, Tanaka, *et al., J. Immunol. 164*:2277, 2000.)

Receptors for PGD_2 include the "DP" receptor, the chemoattractant receptor-homologous molecule expressed on TH2 cells ("CRTH2"), and the "FP" receptor. These receptors are G-protein coupled receptors activated by PGD_2. PGD_2 is a non-selective agonist at the FP receptor. (Abramovitz, *et al., Biochimica et Biophysica Acta 1483*:285-293, 2000.)

Abramovitz, *et al.*, U.S. Patent No. 5,958,723 and Boie, *et al., Journal of Biological Chemistry 270*:18910-18916, 1995, describe the cloning and characterization of the human DP receptor. These references also indicate that PGD_2 activates the DP receptor.

Abe, *et al., Gene 227*:71-77, 1999, Nagata, *et al., FEBS Letters 459*:195-199, 1999, and Nagata, *et al., The Journal of Immunology 162*:1278-1286, 1999, describe CRTH2 and its expression on different cells including human T-helper cells, basophils, and eosinophils. Hirai, *et al., J. Exp. Med. 193*:255-261, 2001, indicates that CRTH2 is a receptor for PGD_2.

SUMMARY OF THE INVENTION

The present invention identifies different activities mediated by eosinophil PGD_2 receptors and features methods measuring the ability of a compound to modulate such activities. Activities mediated by eosinophil PGD_2 receptors include those associated with CRHT2 and those associated with the DP receptor. Activities identified herein as associated with eosinophil CRHT2 include a change in cell morphology, degranulation, and a specific chemokinetic effect. Activities identified herein as associated with the eosinophil DP receptor include resistance to apoptosis.

Measuring the ability of a compound to modulate a PGD_2 receptor activity can be performed quantitatively or qualitatively. Compounds modulating PGD_2 receptor activity include agonists, antagonists and allosteric modulators.

Thus, a first aspect of the present invention features a method that measures the effect of a test compound on either apoptosis or degranulation as a measure of the ability of the compound to modulate a PGD_2 receptor activity. The method employs eosinophil cells.

Another aspect of the present invention describes a method of assaying the ability of a test compound to modulate CRTH2 activity using a compound identified as binding to CRTH2. The method comprises the steps of: (a) identifying a compound that binds to human CRTH2; (b) providing the compound to an eosinophil; and (c) measuring eosinophil morphology, chemokinesis under conditions distinguishing chemokinesis from chemotactic ability, or degranulation.

Another aspect of the present invention describes a method of assaying the ability of a test compound to modulate CRTH2 activity involving the use of a CRTH2 agonist. The method comprises the steps of: (a) providing the test compound and an CRTH2 agonist to an eosinophil, and (b) measuring either eosinophil morphology, chemokinesis under conditions distinguishing chemokinesis from chemotactic ability, or degranulation.

Other features and advantages of the present invention are apparent from the additional descriptions provided herein including the different examples. The provided examples illustrate different components and methodology useful in practicing the present invention. The examples do not limit the claimed invention. Based on the present disclosure the skilled artisan can identify and employ other components and methodology useful for practicing the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates PGD_2 receptor expression on human eosinophils. RT-PCR was performed on total RNA isolated from purified human eosinophils using CRTH2 or DP-specific primers. The RT-PCR product was revealed, by Southern blot, using a CRTH2-specific (top panel) or DP-specific (lower panel) radioactive probe. RNA from HEK cells expressing recombinant CRTH2 or DP receptor was used as a positive control in lane 1. RT-PCR using 18S ribosomal RNA-specific primers was conducted in parrallel to ensure that equivalent amounts of RNA were used between each donor (data not shown). The bands seen are derived from mRNA and not genomic DNA since no signal is detected in absence of reverse transcriptase. Results from two out of four donors tested are shown.

Figure 2 illustrates a rapid change in eosinophil morphology induced by PGD_2. Purified human eosinophils were incubated for 15 minutes with various agents in a 24-well dish. Cells were then magnified 200-times using an inverted microscope. a, vehicle-treated eosinophils. b, eosinophils treated with 10 nM PGD_2. c, 1 μM BW245C (a DP-selective agonist) d, 10 nM 13,14-dihydro-15-keto-PGD_2 (DK- PGD_2). e, 100 nM platelet-activating factor; PAF. f, 1 ng/ml of interleukin-5; IL-5. A representative experiment from 20 donors tested is shown.

Figure 3 illustrates the effect of PGD_2 on eosinophil chemokinesis. Purified human eosinophils were treated for 5 minutes with various agents prior to being placed in the upper chamber of a chemotactic unit. No chemoattractant was added to the lower chamber in order to simply measure chemokinesis. After two hours, the number of cells that transmigrated to the lower chamber was evaluated with an hematocytometer. Chemokinesis efficiency is expressed as the number of transmigrating cells with the agent divided by the number of transmigrating cells with vehicle only (fold-increase chemokinesis over background). Lane 1, vehicle treated eosinophils. Lane 2, eosinophils were treated with 100 nM PGD_2, lane 3 with 1 μM BW245C, lane 4 with 100 nM DK- PGD_2, lane 5 with 100 nM of platelet activating factor, lane 6 with 1 ng/ml of interleukin-5 and lanes 7-8-9 with 1 μM of the indicated compounds. For each experiment, each condition was tested in two independent wells. The mean response is indicated by a dash. The effect of PGD_2 at 100 nM is significant with a probability of < 0.001 in repeated measures ANOVA followed by paired t-tests.

Figure 4 illustrates the ability of PGD_2 to trigger eosinophil degranulation. Purified human eosinophils were treated for 1 hour with various

agents. The amount of ECP released in the media was then determined by radioimmunoassay. Lane 1, vehicle treated cells. Lane 2, eosinophils were treated with 100 nM PGD_2, lane 3 with 1 μM BW245C, lane 4 with 100 nM DK-PGD_2, lane 5 with 100 nM of platelet activating factor, lane 6 with 1 ng/ml of interleukin-5 and lanes 7-8-9 with 1 μM of the indicated compounds. The values correspond to the amount of ECP detected under the various conditions minus the value obtained with the vehicle treated cells (amount of ECP over background). For each experiment, each condition was tested in duplicate. The mean response is indicated by a dash. The effect of PGD_2 at 100 nM was tested on 11 donors ($p<0.0003$ in t-test) while the effect of DK- PGD_2 at 100 nM was tested on 8 donors ($p<0.01$). The value presented for PAF is the mean of six independent experiments ($p<0.01$).

Figure 5 illustrates the ability of PGD_2 to increase the survival of eosinophils in culture. Purified human eosinophils were maintained in culture in the presence of various agents for 36 hours. The cells were then harvested and the extent of apoptosis was evaluated by flow cytometry (Annexin V/propidium iodide staining). Cells that have not reached the stage of late apoptosis (thus not positive for both annexin V and propidium iodide staining) were considered to be alive. Lane 1, vehicle treated cells. Lane 2-9, eosinophils treated with 1 μM of the indicated compounds except lane 6 where interleukin-5 was used at 1 ng/ml. The values correspond to the percentage of non-late apoptotic eosinophils in the treated population minus the percentage of non-late apoptotic eosinophils in the vehicle treated population. The mean response is indicated by a dash. The effect of PGD_2 at 1 μM was tested on 7 donors ($p<0.1$ in t-test) while the effect of BW245C at 1 μM was tested on 9 donors ($p<0.0005$).

DETAILED DESCRIPTION OF THE INVENTION

Identifying different effects mediated by eosinophil PGD_2 receptor activation provides for indicators that may be measured to evaluate the ability of a compound to modulate eosinophil PGD_2 receptor activity and provides information concerning the physiological effects of PGD_2 receptor activation. Information concerning the physiological effects of PGD_2 receptor activation can be used to help evaluate the importance of inhibiting a PGD_2 receptor activity.

Compounds modulating eosinophil PGD_2 receptor activity have a variety of different uses including utility as a tool to further study PGD_2 receptor activity and as an agent to achieve a beneficial effect in a patient. Modulating PGD_2

receptor activity includes evoking a response at the receptor and altering a response evoked by a PGD_2 receptor agonist or antagonist.

Beneficial effects of modulating PGD_2 receptor activity include achieving one or more of the following in a patient: the treatment or prevention of an inflammatory disease such as asthma, treatment or prevention of allergic rhinitis or arthritis; and the treatment or prevention of a sleep disorder. A patient is a mammal, preferably a human. Reference to patient does not necessarily indicate the presence of a disease or disorder. The term patient includes subjects treated prophylactically and subjects afflicted with a disease or disorder.

Selective agonists or antagonists that mimic or block PGD_2 actions at the DP receptor, CRTH2 and/or FP receptor may have utility in the treatment of disease states or diseases including but not limited to allergic rhinitis and other allergic conditions in which mast cells, eosinophils, TH2 cells and other immune cells express the DP receptor, CRTH2, and/or FP receptor, or produce PGD_2. Additional examples of therapeutic applications include one or more of the following: sleep disorders; glaucoma; osteoporosis; modulators may be useful as cytoprotective, analgesic or anti-inflammatory agents; modulators inhibiting platelet aggregation may be useful for treating vascular disease, prevention of post-injury blood clotting, rejection in organ transplant and by-pass surgery, congestive heart failure, pulmonary hypotension and Raynaud's disease.

Eosinophil PGD_2 Receptor and Inflammation

Eosinophils were found to express the DP receptor and CRTH2. The different effects mediated by PGD_2 at these receptors appear to assist the inflammation response. Pharmacological blockade of PGD_2-mediated events at both the DP receptor and CRTH2 may reduce damage caused by eosinophils at an inflammation site.

In allergic situations, PGD_2 is released by mast cells and may facilitate entry into the inflammation site through DP-mediated vasodilation/extravasation of eosinophils as well as other circulating leukocytes. (Mantovani, *et al.*, *Lancet 343*:1499, 1994). The entry of eosinophils into the allergic site would be stimulated by the pro-chemokinetic activity of PGD_2 through CRTH2.

The anti-apoptotic and degranulation effects of PGD_2 on eosinophils appear to be playing a factor in inflammation. The survival of resident eosinophils

would be prolonged by the anti-apoptotic effect of PGD_2 acting through the DP receptor.

Eosinophil degranulation triggered by PGD_2 activation through CRTH2 causes the release of granule-derived proteins. The effects of granule proteins include cytotoxicity at the bronchial epithelium, an increase in nonspecific bronchial hyperreactivity and impaired ciliary function.

PGD_2 Receptor Assays

Different types of assays formats can be employed making use of the activities identified herein as associated with the eosinophil DP receptor or the eosinophil CRHT2. Examples of such formats include:

(1) Measuring the ability of a compound to affect apoptosis or degranulation;

(2) Identifying a compound that binds to the human eosinophil CRHT2 and then testing the ability of the compound to affect eosinophil morphology, chemokinesis under conditions distinguishing chemokinesis from chemotactic ability, or degranulation; and

(3) Screening for a CRHT2 antagonist using a CRHT2 agonist and measuring the ability of a test compound to modulate changes in eosinophil morphology, chemokinesis under conditions distinguishing chemokinesis from chemotactic ability, or degranulation produced by the agonist.

Measuring the effect of a compound on apoptosis or degranulation provides an overall measure of the effect of the compound on DP receptor or CRTH2 activity. Measuring apoptosis or degranulation also provides a direct measure on activities that it would be desirable to inhibit.

In an embodiment of the present invention, a binding assay is employed to select for compound binding to a prostaglandin D_2 receptor prior to an apoptosis or degranulation assay. Assays measuring the ability of a compound to bind to a DP receptor or CRTH2, employ a DP receptor or CRTH2 polypeptide comprising a PGD_2 binding site. DP receptor and CRTH2 polypeptides include full-length human receptors and functional derivatives thereof, fragments containing a PGD_2 binding site, and chimeric polypeptides comprising such fragments. A chimeric polypeptide comprising a fragment that binds PGD_2 also contains one or more polypeptide regions not found in a human DP receptor or CRTH2.

Preferably, assays measuring PGD_2 binding employ full length human DP receptor or CRTH2. The human DP receptor is described by Abramovitz, *et al.* U.S. Patent No. 5,958,723. Human CRTH2 is described by Nagata, *et al., The Journal of Immunology 162*:1278-1286, 1999, and Gen-Bank Accession No. AB00535.

PGD_2 receptor amino acid sequences involved in PGD_2 binding can be identified using labeled PGD_2 and different PGD_2 receptor fragments. Different strategies can be employed to select fragments to be tested to narrow down the binding region. Examples of such strategies include testing consecutive fragments about 15 amino acids in length starting at the N-terminus, and testing longer length fragments. If longer length fragments are tested, a fragment binding PGD_2 can be subdivided or mutated to further locate the PGD_2 binding region. Fragments used for binding studies can be generated using recombinant nucleic acid techniques.

Binding assays can be performed using recombinantly produced PGD_2 receptor polypeptides present in different environments. Such environments include, for example, cell extracts and purified cell extracts containing a PGD_2 receptor polypeptide expressed from recombinant nucleic acid or naturally occurring nucleic acid and also include, for example, the use of a purified PGD_2 receptor polypeptide produced by recombinant means or from naturally occurring nucleic acid which is introduced into a different environment.

The ability of a compound to antagonize PGD_2 receptor activity can be evaluated using a PGD_2 agonist able to produce receptor activity and then measuring the ability of one or more test compounds to alter such activity. Agonists that can be employed include those able to stimulate both DP receptor activity and CRHT2 activity and those selective for DP receptor activity or CRHT2 activity. Examples of different types of agonists are PGD_2 which acts at both the DP receptor and CRHT2; 13-14-dihydro-15-keto-PGD_2 which is specific for CRTH2; and BW245C which is specific for the DP receptor.

The effectiveness of an antagonist to alter PGD_2 receptor activity can be evaluated by comparing PGD_2 receptor activity in the presence of the agonist with such activity in the presence of the agonist and antagonist. Different types of assay formats can be employed. For example, a control experiment involving an agonist and a test experiment involving the agonist and a test compound can be performed at the same or at different times.

Techniques for measuring apoptosis, morphology, chemokinesis under conditions distinguishing chemokinesis from chemotactic ability, and degranulation are well known in the art. Changes in morphology can be measured visually with the aid of a microscope, such as by scoring cells with irregular shapes. Techniques for measuring morphology include those described in the Examples provided below.

Apoptosis is a type of cell death that is programmed by the cell. Techniques for measuring apoptosis include those described in the Examples provided below.

Chemokinesis is an increase in cell mobility that is brought about by a reagent in the absence of chemical gradient. Techniques for measuring chemokinesis include those described in the Examples provided below.

Degranulation results in the release of granule-derived proteins, such as the major basic protein, the eosinophil cationic protein, eosinophil-derived neurotoxin, and eosinophil peroxidase. Techniques for measuring degranulation include those described in the Examples provided below.

Dosing For Therapeutic Applications

Guidelines for pharmaceutical administration in general are provided in, for example, *Remington's Pharmaceutical Sciences 18th Edition*, Ed. Gennaro, Mack Publishing, 1990, and *Modern Pharmaceutics 2nd Edition*, Eds. Banker and Rhodes, Marcel Dekker, Inc., 1990, both of which are hereby incorporated by reference herein.

PGD_2 receptor active compounds having appropriate functional groups can be prepared as acidic or base salts. Pharmaceutically acceptable salts (in the form of water- or oil-soluble or dispersible products) include conventional non-toxic salts or the quaternary ammonium salts that are formed, *e.g.*, from inorganic or organic acids or bases. Examples of such salts include acid addition salts such as acetate, adipate, alginate, aspartate, benzoate, benzenesulfonate, bisulfate, butyrate, citrate, camphorate, camphorsulfonate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, fumarate, glucoheptanoate, glycerophosphate, hemisulfate, heptanoate, hexanoate, hydrochloride, hydrobromide, hydroiodide, 2-hydroxyethanesulfonate, lactate, maleate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, oxalate, pamoate, pectinate, persulfate, 3-phenylpropionate, picrate, pivalate, propionate, succinate, tartrate, thiocyanate, tosylate, and undecanoate; and base salts such as ammonium salts, alkali metal salts such as sodium and potassium

salts, alkaline earth metal salts such as calcium and magnesium salts, salts with organic bases such as dicyclohexylamine salts, N-methyl-D-glucamine, and salts with amino acids such as arginine and lysine.

PGD_2 receptor active compounds can be administered using different routes including oral, nasal, by injection, and transmucosally. Active ingredients to be administered orally as a suspension can be prepared according to techniques well known in the art of pharmaceutical formulation and may contain microcrystalline cellulose for imparting bulk, alginic acid or sodium alginate as a suspending agent, methylcellulose as a viscosity enhancer, and sweeteners/flavoring agents. As immediate release tablets, these compositions may contain microcrystalline cellulose, dicalcium phosphate, starch, magnesium stearate and lactose and/or other excipients, binders, extenders, disintegrants, diluents and lubricants.

When administered by nasal aerosol or inhalation, compositions can be prepared according to techniques well known in the art of pharmaceutical formulation. Such techniques can involve preparing solutions in saline, employing benzyl alcohol or other suitable preservatives, absorption promoters to enhance bioavailability, fluorocarbons, or other solubilizing or dispersing agents.

Routes of administration include intravenous (both bolus and infusion), intraperitoneal, subcutaneous, topical with or without occlusion, and intramuscular. Injectable solutions or suspensions known in the art include suitable non-toxic, parenterally-acceptable diluents or solvents, such as mannitol, 1,3-butanediol, water, Ringer's solution and isotonic sodium chloride solution. Dispersing or wetting and suspending agents, include sterile, bland, fixed oils, such as synthetic mono- or diglycerides; and fatty acids, such as oleic acid.

Rectal administration in the form of suppositories, include the use of a suitable non-irritating excipient, such as cocoa butter, synthetic glyceride esters or polyethylene glycols. These excipients are solid at ordinary temperatures, but liquidify and/or dissolve in the rectal cavity to release the drug.

Suitable dosing regimens for therapeutic applications can be obtained taking into account factors well known in the art including age, weight, sex and medical condition of the patient; the severity of the condition to be treated; the route of administration; the renal and hepatic function of the patient; and the particular compound employed.

Optimal precision in achieving concentrations of drug within the range that yields efficacy without toxicity requires a regimen based on the kinetics of the

drug's availability to target sites. This involves a consideration of the distribution, equilibrium, and elimination of a drug. The daily dose for a patient is expected to be between 0.01 and 1,000 mg per adult patient per day.

Examples

Examples are provided below to further illustrate different features of the present invention. The examples also illustrate useful methodology for practicing the invention. These examples do not limit the claimed invention.

Example 1: Material and Methods

This example illustrates different reagents and techniques.

Reagents

PGD_2, fluprostenol and PGE_2 were obtained from Biomol Research Laboratories, (Plymouth Meeting, PA). BW245C, 13,14-dihydro-15-keto-PGD_2 (DK- PGD_2) and latanoprost (free acid) were from Cayman Chemical (Ann Arbor, MI). Platelet activating factor (PAF) was from Sigma (St-Louis, MO). Recombinant human interleukin-5 was produced using a baculovirus system and purified by FPLC. (Brown, *et al.*, *Protein Expr. Purif.* 6:63, 1995.)

Eosinophil Purification

Circulating eosinophils were isolated from heparinized venous blood from normal volunteers. Erythrocytes were removed by addition of Dextran to a final concentration of 0.9% (Dextran T500 from Pharmacia prepared as a 6% stock in 0.9% saline solution). After a 45 minute incubation at room temperature, the leukocytes in the plasma fraction were collected by centrifugation (4 °C, 300xg, 10 minutes), and resuspended in Hank's balanced salt solution (HBSS without calcium and magnesium).

A density step gradient was generated by placing 20 ml of Ficoll-Paque™ (Pharmacia) under 30 ml of resuspended cells. The gradient was centrifuged (4 °C, 400xg, 30 minutes) and the pellet containing the granulocytes was resuspended in 10 ml of water for 15 seconds to lyse any residual erythrocytes. The hypotonic lysis was stopped by the addition of 40 ml of HBSS.

The cells were then centrifuged (4 °C, 300xg, 10 minutes), washed once with 50 ml of HBSS and resuspended in Dulbecco phosphate buffer saline (PBS

without calcium and magnesium from GIBCO-BRL) at a concentration of 1 x 10^9 cells per ml. An equal volume of CD16 magnetic beads (Milteny Biotec) was added and incubated at 4 °C for 30 minutes. At the end of the incubation, the volume was brought to 1 ml with PBS (without Ca^{+2} and Mg^{+2}) and applied to a CS separation column placed in the magnetic field of a MACS separator (Milteny Biotec).

The CD16+ neutrophils were retained in the column while a >95% pure fraction of CD16- eosinophils eluted from the column. The purity of the eosinophil fraction was evaluated by flow cytometry (CELL-DYN 3700 System) based on size, complexity, granularity and lobularity.

RT-PCR and Southern Blot

Total RNA was obtained from isolated eosinophils (>95% pure) by using a total RNA isolation kit (Rneasy kit, Qiagen) and treated with DNAse (Gibco-BRL) prior to reverse transcription (Gene Amp kit, Perkin Elmer). Amplification of DP receptor by PCR (Advantage GC kit, Clontech) used the following primers: DP sense, 5'-ACAACTCGTTGTGCCAAGCC (SEQ. ID. NO. 1); DP antisense, 5'-GCATCGCATAGAGGTTGCGC (SEQ. ID. NO. 2);. CRTH2 sense, 5'-CTACAATGTGCTGCTCCTGAAC (SEQ. ID. NO. 3); CRTH2 antisense, 5'-CAGGTGAGCACGTAGAGCAC (SEQ. ID. NO. 4). The PCR reaction (50 µl) included a denaturation step (94 °C, 1 minute) and 35 cycles of PCR (94 °C, 30 seconds; 55 °C, 30 seconds; 68 °C, 1 minutes).

PCR reactions were electrophoresed in agarose gels and transferred to nylon N+Hybond membrane (Amersham). The blot was hybridized with a ^{32}P-labeled DNA fragment encoding the full-length hCRTH2 or hDP receptor in ExpressHyb solution (Clontech) overnight at 68 °C. The blot was washed twice in 2X SSC (at 65 °C) and twice in 0.2X SSC (at 65 °C) for 30 minutes each. Results were revealed by autoradiography.

In Situ Hybridization

Freshly isolated eosinophils (2 x 10^5) were layered onto a poly-D-lysine coated glass slide by centrifugation (Cytospin). Cells were then fixed in 4% paraformaldehyde solution prepared in PBS (pH of 7.4) for 20 minutes at room temperature. The slides were then processed as follows: 2 minutes in 3X PBS, 2 times 2 minutes in 1X PBS and incubations of 5 minutes in 50%, 70%, 95% and 100% aqueous ethanol solutions. Slides were air dried and stored at –80 °C.

The slides were thawed to room temperature and washed for 5 minutes in diethylpyrocarbonate (DEPC)-treated water and twice in PBS. The sections were treated with 1.0 μg/ml proteinase K in 100 mM Tris, pH 8.0, 50 mM EDTA for 10 minutes at 37 °C and washed for 5 minutes in DEPC-treated water. The slides were then washed in 0.1 M triethanolamine, pH 8.0 (TEA) for 5 minutes and washed again for 10 minutes in TEA with 0.25% acetic anhydride. Finally, the sections were washed twice for 5 minutes in 2x SSC.

A 398 bp fragment representing the 5' terminal end of the human DP receptor cDNA was amplified by PCR and subcloned into the PCR II dual promoter vector (Invitrogen). The plasmid was linearized using either Xho I or Spe I and digoxigenin-labeled (DIG) riboprobes were synthesized using the DIG-RNA labeling kit from Boehringer Mannheim. The riboprobes were diluted in 75% hybridization buffer (75% formamide, 3x SSC, 1x Denhardt's, 0.2 mg/ml yeast tRNA, 50 mM sodium phosphate, 10% dextran sulfate) and layered onto the cytospin slides. The slides were covered with parafilm and left to hybridize for 16 hours at 55 °C in a humidified (75% formamide) chamber. The parafilm was then removed by soaking the slides in 2x SSC for 30 minutes. The sections were then treated with RNase A (40 μg/ml in 10 mM Tris, pH 8.0, 500 mM NaCl) for 45 minutes at 37 °C. The slides were washed in 2x SSC, 1x SSC, 0.5x SSC (for 10 minutes each at room temperature) and 0.1x SSC (45 minutes at 60°C).

Colorimetric detection of the DIG-labeled riboprobe was done using an alkaline phosphatase-linked anti-DIG antibody (Boehringer Mannheim). All subsequent steps were carried out at room temperature. The slides were washed in detection buffer (DB; 100 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween), incubated with SuperBlock buffer (Biogenex) for 10 minutes and then incubated for 2 hours with the antibody (1:75 dilution) in DB and then washed three times in DB. The chromagen solution (Fast Red, Sigma biochemicals) was then added and the slides were left to incubate for 30 minutes. The reaction was stopped by washing in 10 mM Tris pH 8.0, 1 mM EDTA. The cells were mounted using SlowFade (Molecular probes) and examined on a fluorescent microscope connected to a CCD camera.

Microscopy

Purified eosinophils were incubated in RPMI 1640 media supplemented with 0.5% fetal bovine serum in the presence of the compound to be tested for 15 minutes in a 24-well dish. Light microscopy was performed with an

inverted Axiovert 25 (Zeiss) and images were obtained with a 35 mm SLR camera (ARIA CONTAX, Kyocera corporation) using Kodak Elite Chrome 160T film.

Eosinophil Chemokinesis

Purified eosinophils were resuspended at 3.0 x 10^6 cells per ml in RPMI 1640 medium supplemented with 0.5% (v/v) fetal bovine serum. Compounds to be tested were added from 1000X concentrated stock solutions to 100 µl of cells in a 1.5 ml centrifuge tube and incubated at room temperature for 5 minutes. 100 µl of treated cells were then added to the top half of a chemotactic chamber (6.5mm Transwell, 3.0 µm polycarbonate membrane from Costar) and 600 µl of RPMI, supplemented with 0.5% (v/v) fetal bovine serum, was added to the bottom chamber. After a 2 hour incubation at 37°C in a CO_2 chamber, the top chamber was discarded and the number of cells that had migrated to the lower chamber was evaluated by counting the cells using an hematocytometer. For each condition tested, the number of migrating eosinophils in two chemotactic chambers was averaged.

Eosinophil Degranulation

Purified eosinophils were resuspended at 3.0 x 10^6 cells per ml in RPMI 1640 medium supplemented with 0.5% (v/v) fetal bovine serum. Compounds to be tested were diluted 1:1000 to their final concentration in 300 µl of cells in a 1.5 ml centrifuge tube. The cells were immediately transferred to a 24-well plate. After an 1 hour incubation at 37 °C in a CO_2 chamber, the cells were removed by centrifugation (4 °C, 300xg, 10 minutes). Eosinophil cationic protein (ECP) in the supernatant was quantified by a double antibody radioimmunoassay (Pharmacia) following the manufacturer's protocol.

In Vitro Eosinophil Apoptosis Assay

Purified eosinophils were resuspended at 2.0 x 10^5 cells per ml in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 2 mM glutamine, and 100 units of penicillin and streptomycin. Compounds to be tested were diluted 1:1000 to their final concentration and the cells were incubated at 37 °C in a CO_2 chamber for 36 hours. The extent of apoptosis in the eosinophil population was evaluated using the TACS™ Annexin-V-FITC apoptosis detection kit (R&D systems). Non-apoptotic cells are not stained with either Annexin-V FITC or propidium iodide. Early apoptotic cells are stained with Annexin-V FITC but not

propidium iodide (green fluorescence). Late apoptotic cells are stained with both Annexin-V FITC and propidium iodide (dual green and red fluorescence). Necrotic cells are only stained with propidium iodide (red fluoresecence). Labeled eosinophils were analyzed in a FACS Calibur system from Becton Dickinson.

Example 2 : Eosinophil Expression of DP and CRTH2

To establish which PGD_2-binding receptors are expressed by human eosinophils, RT-PCR was performed on total RNA from human eosinophil (>95% purity). The identity of the PCR products was confirmed by Southern blot detection using DP receptor and CRTH2-specific probes.

CRTH2 mRNA was detected in eosinophils from four donors while DP mRNA was detected in only two of the four donors (Figure 1). The identity of the cell type expressing DP receptor as an eosinophil was confirmed by *in situ* hybridization. DP antisense hybridized only to cells showing the characteristic bi-lobal nucleus of eosinophils.

Example 3: PGD_2 Induced a Change in Eosinophil Morphology Through CRTH2

PGD_2 (<10 nM) induced dramatic changes in cell morphology within minutes. Vehicle-treated eosinophils were spherical and only weakly adhered to the culture dish. In contrast, eosinophils treated with PGD_2 become flat, assumed an Amoeba-like shape and showed round structures which may represent secretory vesicles (panel 2b). This effect was seen on the majority of eosinophils from all donors analysed (n=20). PGD_2-treated eosinophils reverted to a spherical shape within six hours. These cells were resistant to morphology changes after a second PGD_2 challenge (data not shown).

The DP receptor selective agonist, BW245C, at concentrations as high as 1 μM did not affect eosinophil shape (panel 2c). In contrast, a CRTH2 selective agonist, DK- PGD_2, induced a morphological change identical to that observed with PGD_2 (panel 2d). Known activators of eosinophils such as platelet activating factor (PAF) (panel 2e) as well as interleukin-5 (Il-5) (panel 2f) also lead to a rapid change in eosinophil morphology. Other prostanoid receptor agonists such as PGE_2 (EP receptors) as well as fluprostenol and latanoprost (FP receptor) did not cause, even at μM doses, any alterations of eosinophil morphology (data not shown). These data suggest that the morphological changes induced by PGD_2 and DK- PGD_2 on eosinophils are mediated through the CRTH2 and not the DP receptor.

Example 4: PGD_2 Increases Eosinophil Chemokinesis Through CRTH2

PGD_2 increased cell motility in the absence of a chemical gradient, a process defined as chemokinesis. PGD_2 was not observed to exert a chemotatic effect. Overall, the data indicates that PGD_2 modulates eosinophil chemokinesis in a DP-independent manner and most likely through the CRTH2.

Chemokinesis was measured by incubating eosinophils with PGD_2 for 5 minutes prior to their loading in the upper chamber of a chemotactic unit lacking a chemoattractant in the lower chamber. PGD_2 at a concentration of 100 mM increased eosinophil chemokinesis by 6-fold compared to cells treated with vehicle only (Figure 3). PGD_2 at concentrations of 10 nM and 1 μM increased eosinophil chemokinesis by a factor of 5 and 9-fold respectively (data not shown). PAF and Il-5 were also able to stimulate eosinophil chemokinesis (Figure 3). (See, Wardlaw, *et al., J. Clin. Invest. 78*:1701, 1986, Schweizer, *et al., J. Leukoc. Biol. 59*:347, 1996.) DK- PGD_2 but not BW245C was effective in stimulating eosinophil chemokinesis (Figure 3). EP and FP receptor agonists, PGE_2, fluprostenol and latanoprost failed to modulate eosinophil migration.

Chemotaxis was measured by adding PGD_2 to the bottom chamber of a chemotactic unit containing eosinophils in the top chamber. In contrast to chemoattractants such as PAF and eotaxin, PGD_2 was not a chemoattractant since it did not attract eosinophils to the lower chamber of the chemotactic unit (data not shown). Eosinophils pre-incubated with PGD_2 (up to 1 μM for 5 minutes to 18 hours) did not have an altered chemotactic response to either PAF or eotaxin (data not shown).

Example 5: PGD_2 Triggers Eosinophil Degranulation Through CRTH2

Degranulation of eosinophils was assayed by challenging freshly isolated eosinophils with PGD_2 and measuring release of the eosinophil cationic protein (ECP) into the media using an ECP-specific radioimmunoassay. PGD_2 at a concentration of 10 to 100 nM significantly increased the release of ECP from eosinophils into the media (Figure 4).

Among the donors, a broad range in PAF- and PGD_2-induced release of ECP is seen. On average, the extent of PGD_2-induced ECP release is about half that seen with PAF. In general the extent of degranulation induced by PGD_2 and PAF paralleled each other. ECP was not released as a result of necrosis as lactate

dehydrogenase (LDH), a marker for necrotic cell lysis, was not detected in the media (data not shown). We also observed the release from eosinophils of another granular protein, EDN, after PGD_2 challenge (data not shown). DK-PGD_2, but not BW245C, significantly increased the release of ECP (Figure 4). FP and EP receptor agonists did not induce ECP release. As seen with eosinophil morphology changes and chemokinesis, PGD_2 induces eosinophil degranulation by a CRTH2-dependent mechanism.

Example 6 : A Selective DP Agonist Delays The Onset Of Apoptosis

The ability of PGD_2 to modulate apoptosis in eosinophils was measured by quantifying the capacity of Annexin V to bind to phosphatidylserine on the outer membrane of apoptotic cells. (Koopman, *et al.*, *Blood 84*:1415, 1994.) Necrotic cell death was determined by propidium iodide uptake. (Darzynkiewicz, *et al.*, *Cytometry 13*:795, 1992.) Annexin V-FITC and propidium iodide staining of eosinophils was evaluated by FACS analysis.

Isolated eosinophils become apoptotic after approximately 12 hours when cultured in RPMI-1640 supplemented with 10% fetal bovine serum. After 48 hours almost all eosinophils were dead (data not shown). Addition of Il-5 or PGE_2 to the media increased the percentage of non-apoptotic eosinophils at 36 hours in culture (Figure 5).

PGD_2 was a weak inhibitor of apoptotic cell death while DK- PGD_2 had no significant effect (Figure 5). In contrast, the DP-specific agonist BW245C significantly increased the percentage of non-apoptotic eosinophil by 17%. The effects of FP agonists, fluprostenol and latanoprost were not significant.

Other embodiments are within the following claims. While several embodiments have been shown and described, various modifications may be made without departing from the spirit and scope of the present invention.

WHAT IS CLAIMED IS:

1. A method of assaying the ability of a test compound to modulate prostaglandin D_2 receptor activity comprising the steps of:

a) providing said test compound to an eosinophil; and

b) measuring the effect of said test compound on either apoptosis or degranulation as a measure of the ability of said test compound to modulate prostaglandin D_2 receptor activity.

2. The method of claim 1, wherein prior to said step (a) said test compound has been identified as able to bind to the DP receptor and said method measures apoptosis.

3. The method of claim 1, wherein said method further comprises providing a DP receptor agonist to said eosinophil and measuring the ability of said test compound to affect apoptosis.

4. The method of claim 1, wherein prior to said step (a) said test compound has been identified as able to bind to CRTH2 and said method measures degranulation.

5. A method of assaying the ability of a test compound to modulate CRTH2 receptor activity comprising the steps of:

a) identifying a compound that binds to a human CRTH2;

b) providing said test compound to an eosinophil; and

c) measuring either eosinophil morphology, chemokinesis under conditions distinguishing chemokinesis from chemotactic ability, or degranulation.

6. The method of claim 5, wherein said step (c) measures eosinophil morphology.

7. The method of claim 5, wherein said step (c) measures chemokinesis.

8. The method of claim 5, wherein said step (c) measures degranulation.

9. A method of assaying the ability of a test compound to modulate CRTH2 activity comprising the steps of:

a) providing said test compound and an CRTH2 agonist to an eosinophil; and

b) measuring either eosinophil morphology, chemokinesis under conditions distinguishing chemokinesis from chemotactic ability, or degranulation.

10. The method of claim 9, wherein said step (c) measures eosinophil morphology.

11. The method of claim 9, wherein said step (c) measures chemokinesis.

12. The method of claim 9, wherein said step (c) measures degranulation.

13. The method of claim 12, wherein said agonist distinguishes CRTH2 from the DP receptor.

14. The method of claim 13, wherein said agonist is 13-14-dihydro-15-keto-PGD_2.

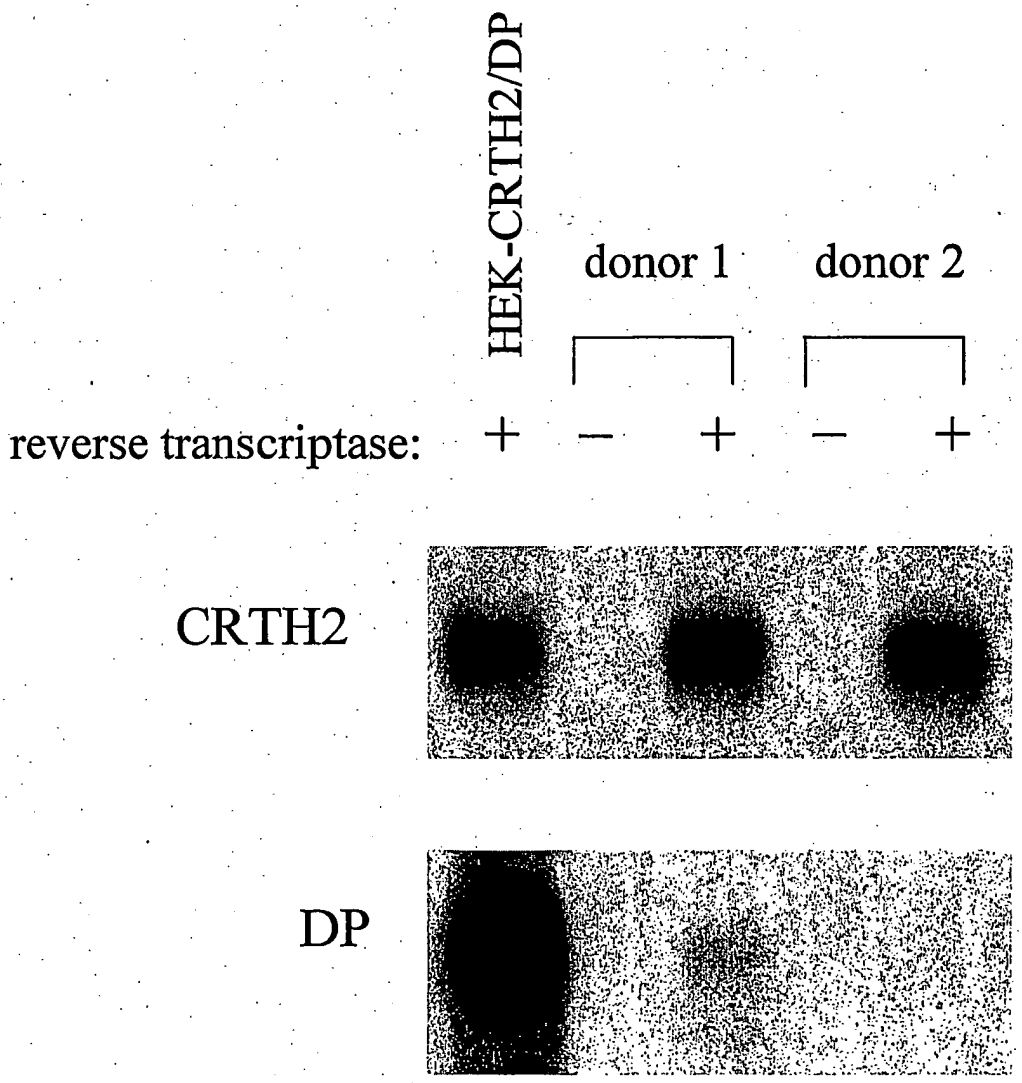


FIG. 1

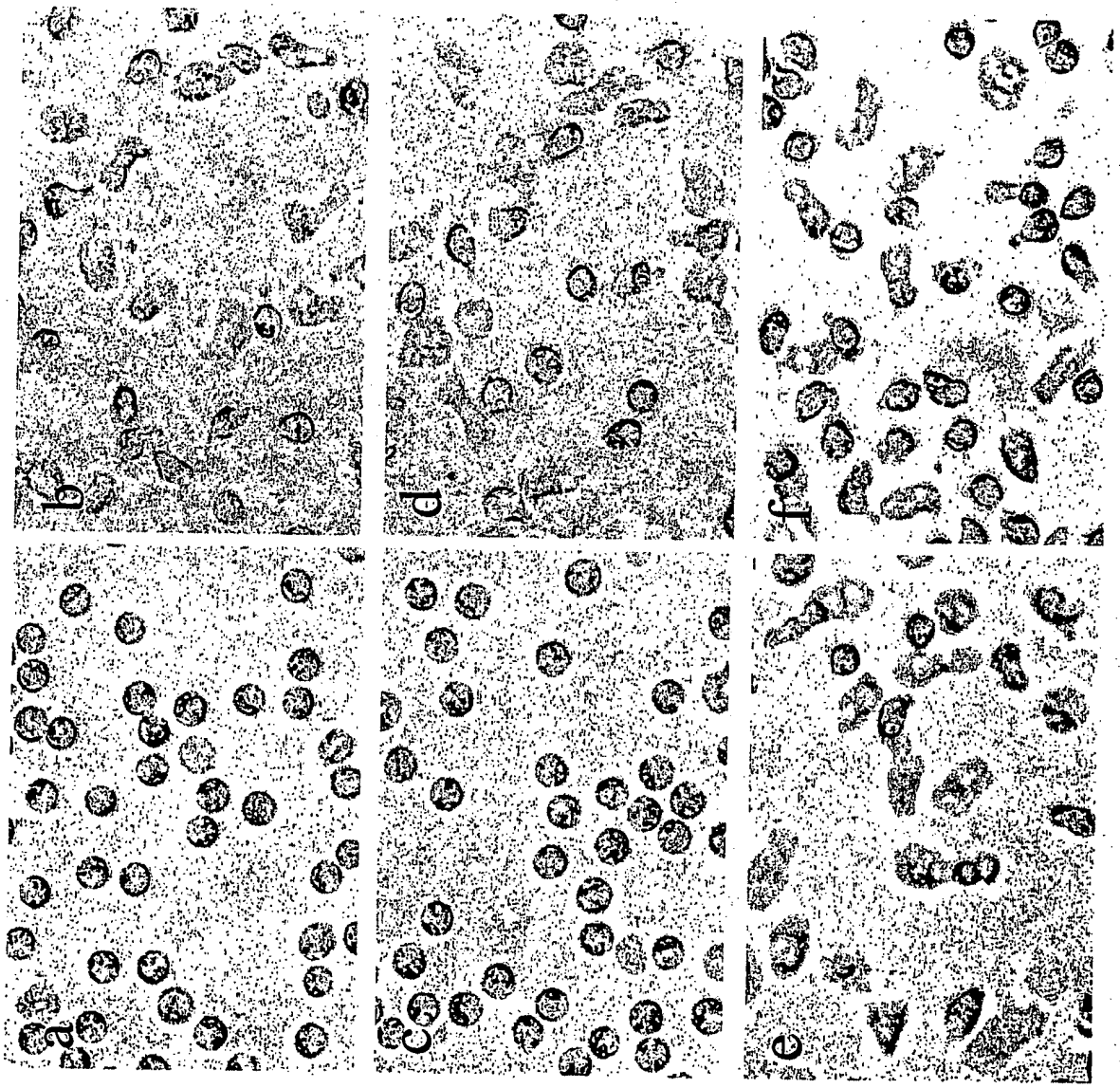
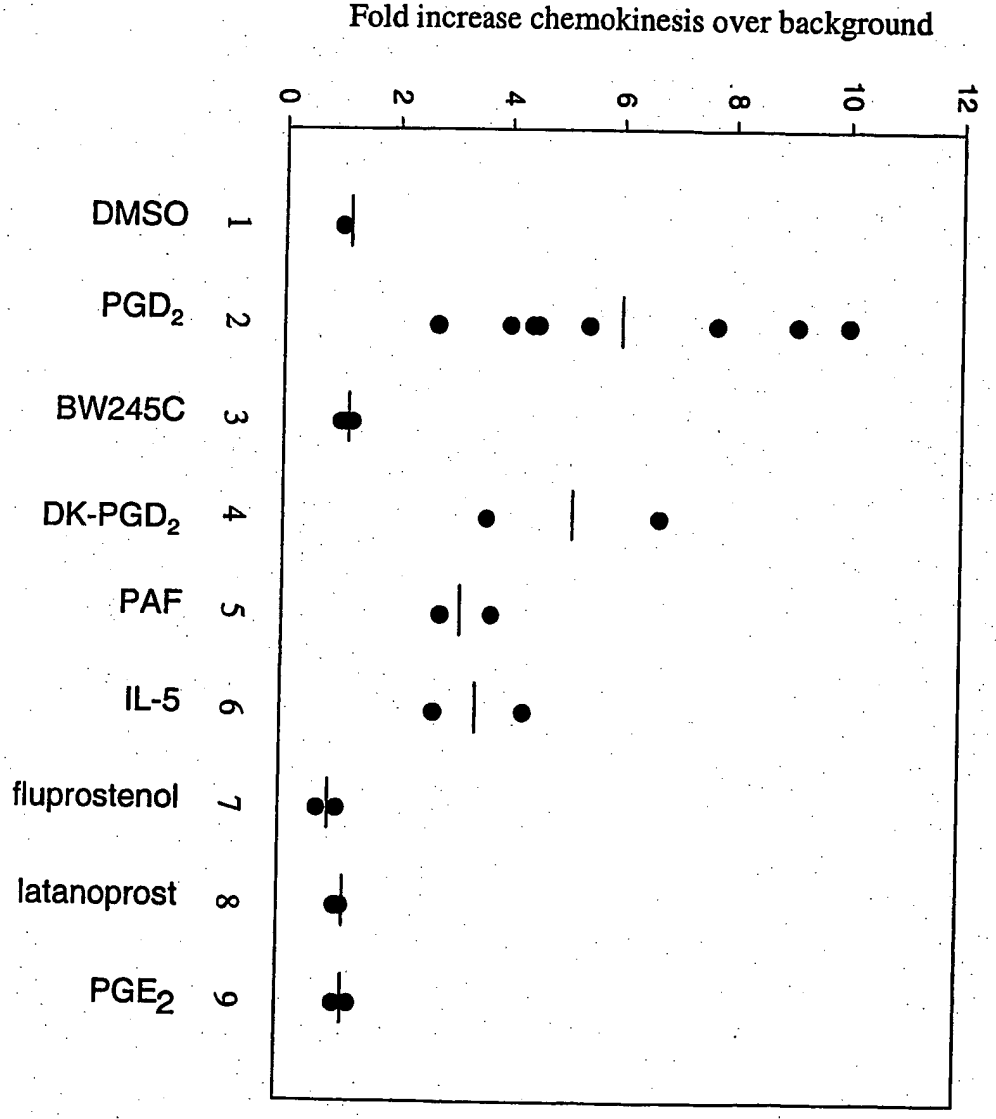


FIG. 2


Fold increase chemokinesis over background
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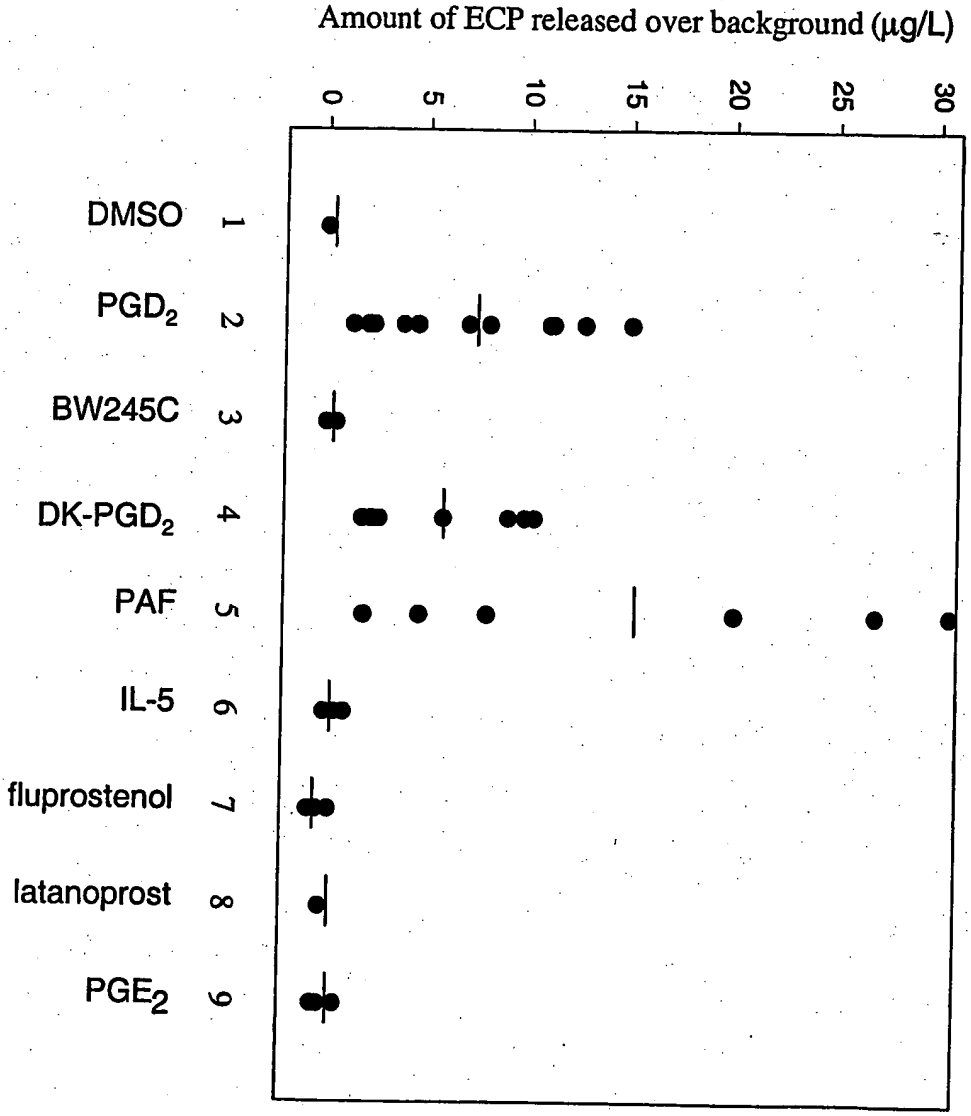
2
4
6
8
10
12
DMSO
1
PGD2
2
BW245C
3
DK-PGD2
4
PAF
5
IL-5
6
fluprostenol
7
latanoprost
8
PGE2
9

FIG. 3


Amount of ECP released over background (µg/L)
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5
10
15
20
25
30
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BW245C 3
DK-PGD2 4
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IL-5 6
fluprostenol 7
latanoprost 8
PGE2 9

FIG. 4

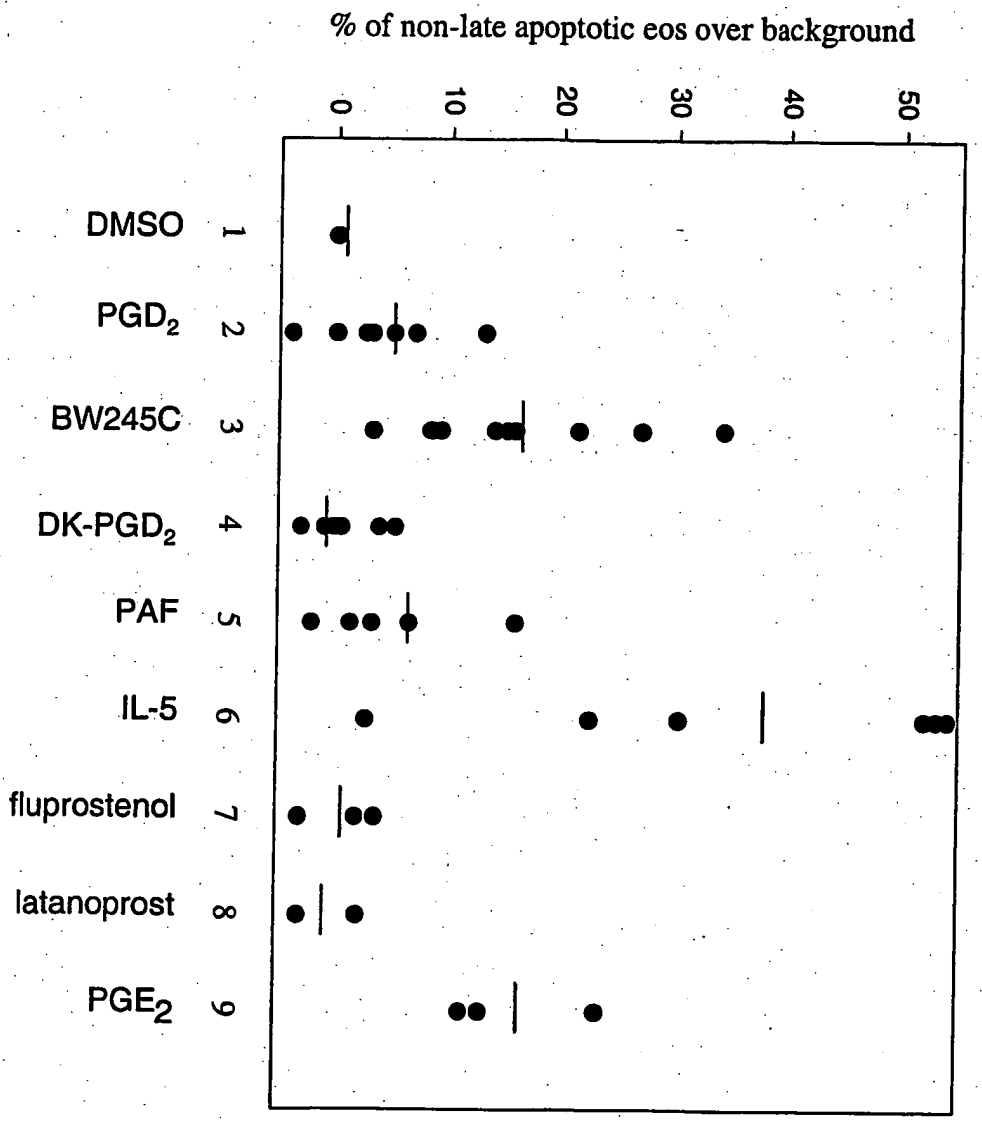
% of non-late apoptotic eos over background
0
10
20
30
40
50
DMSO
1
PGD2
2
BW245C
3
DK-PGD2
4
PAF
5
IL-5
6
fluprostenol
7
latanoprost
8
PGE2
9

FIG. 5

SEQUENCE LISTING

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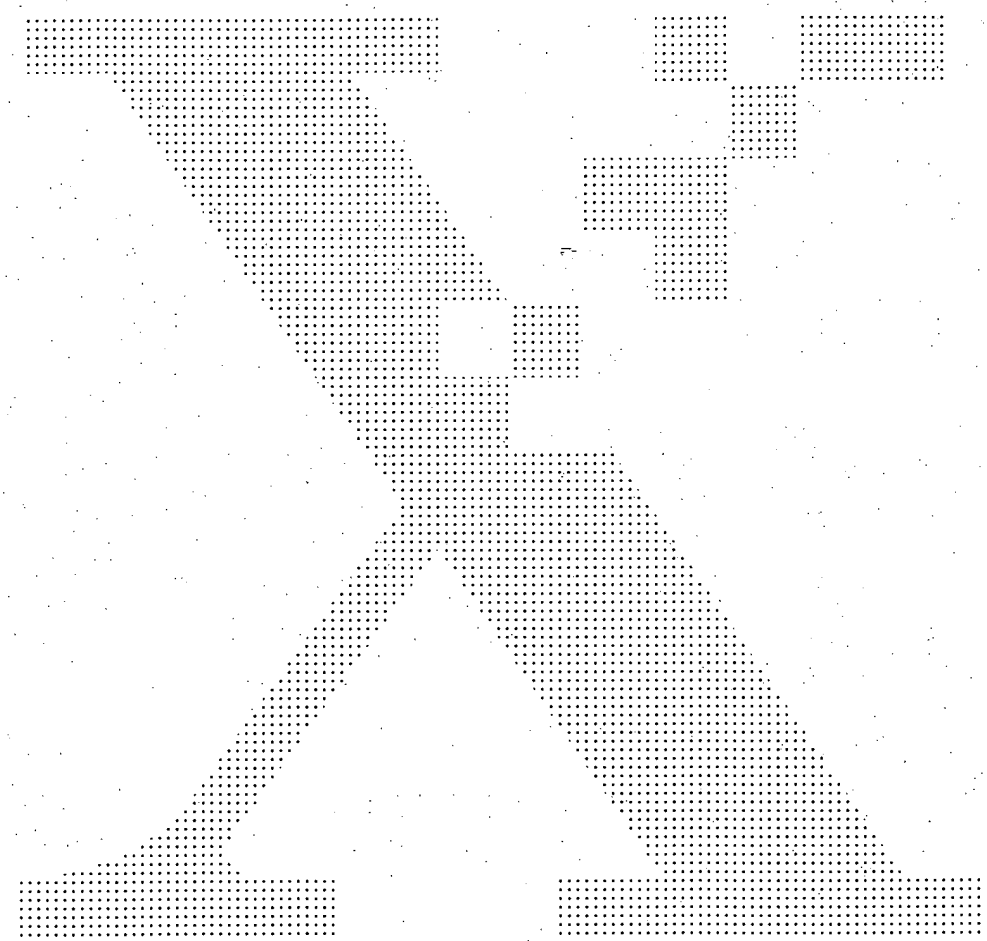

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(54) Title: A PSEUDO-SEQUENCE METHOD FOR COMPARING 7TM RECEPTORS WITH RESPECT TO THE PHYSICOCHEMICAL PROPERTIES OF THEIR BINDING SITES

(57) Abstract: A pseudo-sequence method for comparing 7TM receptors with respect to the physicochemical properties of their binding sites, the method comprising the steps of: (i) optionally, aligning part of or all of the amino acid sequence of the first 7TM receptor with part of or all or the amino acid sequence of the one or more further 7TM receptors, (ii) selecting, in a sequential or non-sequential order, at the most 12 amino acid residues per helix and/or extracellular loops, which are involved in one or more binding sites of each 7TM receptor. (iii) forming a pseudo-sequence comprising at the most 50 amino acid residues from the selected sequential or non-sequential amino acid residues, (iv) for each 7TM receptor assigning one or more physicochemical descriptors to the amino acid residues of the selected amino acid pseudo-sequence involved in one or more binding sites, (v) optionally, for each 7TM receptor mathematically manipulating the physicochemical descriptors of step (iv) to obtain a simplified measure of the physicochemical properties of the binding site. (vi) for each 7TM receptor generating a similarity score as defined herein by comparing the physicochemical descriptor or, if relevant, the simplified measure for the first 7TM receptor with the physicochemical descriptors or, if relevant, the simplified measures for the one or further 7TM receptors, (vii) optionally, ranking the 7TM receptors with respect to the physicochemical properties of their binding sites according to the similarity scores obtained in step vi)

WO 2004/079625 A2

A pseudo-sequence method for comparing 7TM receptors with respect to the physicochemical properties of their binding sites

Field of the invention

The present invention relates to comparisons of 7TM receptor proteins with respect to the physicochemical properties of amino acid residues in the binding site without information from ligands interacting with the receptor.

Background

7TM receptor proteins constitute the largest family of biological targets for current drugs. Drug development of ligands for these receptors has not been able to benefit from efficient structure-based drug design technologies due to the lack of detailed structural information. Determination of the exact structure of the binding site of transmembrane receptors is not generally amenable to X-ray crystallography or NMR as for soluble proteins, due to the inherent difficulties in producing large amounts of active membrane protein and in the case of X-ray crystallography producing crystals of these. Accordingly, the 3D structure of bovine rhodopsin is the only receptor that so far has been solved by X-ray crystallography at atomic resolution (Palczewski. K. et al, *Science*, (2000) **289**, 739-745).

Despite the low sequence similarities, homology models of other 7TM receptors of the Class A type have been derived based on the bovine rhodopsin structure. Identification of binding sites and binding modes of ligands to 7TM receptors are supported by combining information from other experimental techniques; for example, site-directed mutagenesis, metal-ion site engineering, substituted cysteine accessibility method, site directed spin labelling and photoaffinity labelling. For references on designing drugs for 7TM receptors or G-protein coupled receptors (GPCRs), see: Drug design strategies for targeting G-protein coupled receptors. Klabunde,T.; Hessler, G. *ChemBioChem* (2002) **3**, 928-944; G-Protein coupled receptors: Models, mutagenesis, and drug design. Bikker, J.A.; Trumpp-Kallmeyer, S.; Humblet, C. *Journal of Medicinal Chemistry* (1998) **41**, 2911-2927; Modelling G-protein coupled receptors for drug design. Flower, D.R. *Biocimica et Biophysica Acta* (1999) **1422**, 207-234; Locating ligand-binding sites in 7TM receptors by protein engineering, T.W. Schwartz. *Curr. Opin. Biotechnol.* (1994) **5**, 434-444.

Calculated similarity scores to infer the overall "global" protein sequence homology are known (Needleman S. & Wunsch C. L. *J. Mol. Biol.* **48**, 444-453 1970; Smith T.F. & Waterman M. *J. Mol. Biol.* (1981) **147**, 195-95; Pearson W.R. & Lipman D.J. *Proc. Natl. Acad. Sci. USA*, (1988) **85**, 2444-48 ; Altschul S.F. *J. Mol. Biol.* (1991) **219**, 555-565; Altschul S.F. *J. Mol. Evol.* (1993) **36**, 290-300). Traditional evolutionary phylogenetic analyses considering the protein "global" sequences are also known. Conventional sequence similarity scores and phylogenetic analysis of protein sequences are based upon statistics on how frequently different amino acids are evolutionary replaced with other amino acids as reported in substitution matrices such as the BLOSUM (derived form blocks of aligned sequences) and PARM (point accepted mutations) series (Henikoff S. & Henicoff J.G. *Proc. Natl. Acad. Sci. USA*, (1992) **89**, 10915-919; Henikoff S. & Henicoff J.G. *Proteins* (1993) **17**, 49-61; Dayhoff M. Schwartz R.M. & Orcutt B.C. *Atlas of protein Sequence and Structure* (1978) **5**, 345-52) and they reflect how nature has happened to replace them during the evolutionary period.

As illustrated in the conventional phylogenetic analysis of the GPR44 (CRTH2) receptor (Figure 1), the following relationships with a number of receptors are revealed according to reference *J. Exp. Med. Volume* (2001) **193**, 255–261.Notably, the AT1 and AT2 receptors are not identified according to this evolutionary relationship model. We will later show how different the relationships of receptors to GPR44 with respect to the ligand-binding properties in the binding site are.

Such evolutionary-based similarity scores of sequences are different from similarity scores based on actual physicochemical properties associated with the individual amino acids. Only in the cases where phylogenetically very closely related proteins are compared, will the analyses yield a similar result.

Other means to compare mono-amine related 7TM receptors based on chemogenomics input have been devised (A novel chemogenomics knowledge-based ligand design strategy – Application to G-protein-coupled receptors, E. Jacoby. *Quant. Struct.-Act. Relat.* (2001) **20**, 115-123).

Sequence similarity (as defined in various substitution matrices) versus chemical sequence similarity will usually not produce comparable similarity scores or identify the same related receptors. In this invention, selected amino acid residues defined as being part of the 7TM receptor binding site and constituting an amino acid pseudo-

sequence are assigned physiochemical descriptors, which are compared and ranked according to a given receptor of interest.

There exists a need for a novel method for comparing 7TM receptors with respect to the physicochemical properties of their binding sites, without using information from a ligand. This is especially important when considering 7TM receptors for which no ligands are known e.g. orphan receptors.

Summary of the invention

An understanding of the physicochemical properties of a binding site will assist in designing ligands that may bind to a receptor. The present invention describes methods for comparing and/or ranking 7TM receptors according to the physicochemical properties of their binding sites, allowing similarities and differences between said 7TM receptors to be identified.

In one embodiment, the method according to the present invention relates to a pseudo-sequence method for comparing a first 7TM receptor with one or more further 7TM receptors with respect to the physicochemical properties of their binding sites, the method comprising the steps of:

i) optionally, aligning part of or all of the amino acid sequence of the first 7TM receptor with part of or all of the amino acid sequence of the one or more further 7TM receptors,

ii) selecting, in a sequential or non-sequential order, at the most 12 amino acid residues per helix, and at the most 12 amino acid residues in one or more extracellular loops, which are involved in one or more binding sites of each 7TM receptor,

iii) forming a pseudo-sequence comprising at the most 50 amino acids from the selected sequential or non-sequential amino acid residues,

iv) for each 7TM receptor assigning one or more physicochemical descriptors to the amino acid residues of the selected amino acid pseudo-sequence involved in one or more binding sites,

v) optionally, for each 7TM receptor mathematically manipulating the physicochemical descriptors of step iv) to obtain a simplified measure of the physicochemical properties of the binding site,

vi) for each 7TM receptor generating a similarity score as defined herein by comparing the physicochemical descriptor or, if relevant, the simplified measure for the first 7TM receptor with the physicochemical descriptors or, if relevant, the simplified measures for the one or further 7TM receptors,

vii) optionally, ranking the 7TM receptors with respect to the physicochemical properties of their binding sites according to the similarity scores obtained in step vi).

In a second embodiment, the present invention describes a method for classifying 7TM receptors according to the physicochemical properties of their binding sites

The invention further relates to drug discovery methods for identifying ligands, which bind to a first 7TM receptor and bind or potentially bind to one or more further 7TM receptors. Additionally, methods are described for identifying a lead compound or a potential lead compound for a 7TM receptor. Furthermore, the present invention describes a drug discovery method for constructing a pharmacophore model for a 7TM receptor.

Importantly, the methods of the present invention can be carried out using only information from the 7TM receptor, i.e. without any information on a ligand or on ligand-receptor interactions.

Detailed description of the invention

The following abbreviations and definitions are used throughout the description.

A glossary of terms used in Medicinal Chemistry or Computational Drug Design is found in *Annual Reports in Medicinal Chemistry*, Volume 33, Division of Medicinal Chemistry of ACS, Academic Press, pages 385-409. For a discussion of the difference between "Leads" and "Drugs", see T.I. Oprea *et al*, *J. Chem. Inf. Comput. Sci.*, 2001, **41**, 1308-1315.

7TM receptor, i.e. a 7-transmembranal (7TM) receptor having seven α-helices that span the cell membrane and are usually coupled to G-proteins. A list of specific 7TM receptors is given herein. The term is used interchangeably with "*receptor*", and one skilled in the art will understand the meaning according to the context.

A "*binding site*" is a region of a biological molecule (e.g. a protein such as a 7TM receptor) to which a ligand may bind. A binding site comprises one or more amino acid residues arranged in a particular geometry so as to provide an environment with a specific arrangement of charged, polar or non-polar regions, which can interact with a ligand. The binding site could represent the region encompassing the entire ligand, or consist of the main ligand-binding site, or be a subsite engaged in interactions with a part of the ligand.

An "*amino acid pseudo-sequence*" is defined as selected amino acid residues in sequential or non-sequential order, involved in one of more ligand binding sites in 7TM receptors. In comparing pseudo-sequences for different 7TM receptors the positions in the pseudo-sequence correspond to the same generic numbers. E.g. a generic pseudo-sequence could be exemplified with amino acids positioned in I-04, I-15, I-20, II-02, II-07, II-21, III-06, III-13,....., VII-02, VII-09.

"*Binding site model*", i.e. a model achieved by computer-assisted molecular design (CAMD) that describes how a ligand binds to a 7TM receptor.

A "*bitmap*" is defined as a string of physicochemical descriptor values used to describe certain chemical features of amino acid residues of interest. A bitmap may be derived from all physicochemical descriptors of the amino acid residues of the binding site or from a simplified measure of the physicochemical descriptors.

"Chemogenomics," i.e. correlation of chemical features of known biologically active compounds (i.e. ligands) with various biological targets. Cf. "Methodologies used for analysis of common drug shapes" G. W. Bemis and M. A. Murcko, *J. Med. Chem.*, 1996, **39**, 2887-2893.

"Computer-assisted drug design (CADD)", i.e. computational techniques used to discover, design, and optimise small organic compounds that are biologically active compounds with a putative use as drugs.

"Design", i.e. application of all techniques leading to the discovery of new chemical entities (e.g. ligands) with specific properties such as affinity for a given receptor.

"Library or chemical library", i.e. collection of ligands that are often produced by parallel synthesis or represent a collection of historic or commercial compounds. Cf. B. A. Bunin *et al, Ann. Rep. Med. Chem*, 1999, 34, 267-286.

"Ligand", i.e. small organic compound that might display affinity for a biological target molecule such as a 7TM receptor. Throughout the specification the term ligand is sometimes used equivalently with the term small organic compound. A person skilled in the art will understand the meaning according to the context.

"Main ligand-binding site", refer to the binding site located between TM-III, IV, -V, -VI, and VII in 7TM receptors which corresponds to where, for example, the main part of retinal is found in the rhodopsin structure and to where ligands have been mapped to bind in a variety of 7TM receptors. Residues, which line this site, are those that are generally involved in ligand binding, and therefore those, which are preferred residues in alignments in the present invention.

"Pharmacophore model", i.e. a model describing the combination of steric and electronic features of a ligand that are necessary for interaction with a specific 7TM receptor which may trigger or block its biological response.

"Physicochemical descriptors" can be experimentally derived and/or theoretically calculated. The descriptors reflect 7TM receptor-ligand interaction features of the amino acid residues, i.e. they may reflect hydrophobic properties, electronic properties, steric properties or hydrogen bonding capabilities and other properties of importance for ligand-protein interactions. Some descriptors can be seen to reflect combinations of such properties, especially combinations of electronic and steric features. The descriptors also include dummy parameters or indicator variables, e.g. 1 and 0, to denote absence or presence of certain properties such as absence or presence of aromatic side chains, hydrophobic side chains, negatively charged side chains, positively charged side chains, polar side chains, hydrogen-bond donating side chains, hydrogen-bond accepting side chains or other selected features

"Receptor model", i.e. a 3-dimensional model of a biological target molecule such as a 7TM receptor based on information from structurally known analogous proteins (homology model) and complementary data such as structure-activity data of ligands or antibodies binding to the biological target molecule and mutational studies.

A *"similarity score"* is a mathematical indicator of the similarity between two (potential) binding sites. Expressions of similarity scores include the Tanimoto coefficient, the Tversky coefficient and the Euclidian distance measure. 7TM receptors having close similarity scores have a high degree of similarity of their (potential) binding sites.

A *"small organic compound"* is intended to indicate a small organic molecule of low molecular weight such as below 1000. The small organic compounds of specific interest in the present context are those that are capable of interacting with a membrane-associated protein such as a 7TM receptor, in such a way as to modify the biological activity thereof.

"Structure-based drug design", i.e. using protein structural information from e.g. X-ray crystallography or NMR spectroscopy to assist in the design of therapeutic compounds – mostly of inhibitors which bind to enzymes. Cf. M.A. Murcko *et al, Ann. Rep. Med. Chem*, 1999, 34, 297-306.

In the method according to the present invention, the amino acid residues of a 7TM receptor are assigned physicochemical descriptors. It is then possible to compare different transmembrane proteins such as 7TM receptors with respect to the physicochemical properties of their binding sites. The process of comparing biological target proteins based on the physicochemical properties of their binding site will be referred to "physicogenomics" herein. The following steps are involved in physicogenomics:

- Sequence search
- Alignments
- Analysis of binding site residues important for ligand binding and recognition
- Comparison of physicochemical properties of binding site

In other words, the method according to the present invention relates to a method for comparing a first 7TM receptor with one or more further 7TM receptors with respect to

the physicochemical properties of their binding sites, the method comprising the steps of:

i) optionally, aligning part of or all of the amino acid sequence of the first 7TM receptor with part of or all of the amino acid sequence of the one or more further 7TM receptors,

ii) selecting, in a sequential or non-sequential order, at the most 12 amino acid residues per helix, and at the most 12 amino acids in one or more extracellular loops, which are involved in one or more binding sites of each 7TM receptor,

iii) forming a pseudo-sequence comprising at the most 50 amino acid residues from the selected sequential or non-sequential amino acid residues,

iv) for each 7TM receptor assigning one or more physicochemical descriptors to the amino acid residues of the selected amino acid pseudo-sequence involved in one or more binding sites,

v) optionally, for each 7TM receptor mathematically manipulating the physicochemical descriptors of step iv) to obtain a simplified measure of the physicochemical properties of the binding site,

vi) for each 7TM receptor generating a similarity score as defined herein by comparing the physicochemical descriptor or, if relevant, the simplified measure for the first 7TM receptor with the physicochemical descriptors or, if relevant, the simplified measures for the one or further 7TM receptors,

vii) optionally, ranking the 7TM receptors with respect to the physicochemical properties of their binding sites according to the similarity scores obtained in step vi).

Importantly, this comparison or ranking can be carried out using only information from the 7TM receptor, i.e. without any information on a ligand. Therefore, the present invention also relates to a method as described herein, wherein the comparison is made without using data related to binding affinity of a ligand to a 7TM receptor.

The present invention may also be used to classify 7TM receptors according to the physicochemical properties of their binding sites. Hence, the present invention

describes a method for classifying 7TM receptors according to the physicochemical properties of their binding sites. This classification may also be carried out using only information from the 7TM receptor, i.e. without any information on a ligand. Hence, the classification may be made without using data related to binding affinity of a ligand to a 7TM receptor.

Method for obtaining lead structures for new 7TM receptors

It is generally recognized that structurally related small organic molecules often bind the same biological target proteins. Cf. "*Do Structurally Similar Molecules Have Similar Biological Activity?*" Yvonne C. Martin, James L. Kofron, and Linda M. Traphagen, J. Med. Chem. (2002) 45, 4350-4358. Conversely, related biological targets often bind the same or similar small organic molecule ligands. Notably, there are exceptions from these general rules – otherwise development of receptor-selective ligands would have been an impossible task.

Having identified which 7TM receptors resemble each other with respect to the physiochemical environment in the binding site as described herein, it is possible to utilise known ligands which interact with one receptor as chemical starting points (so called chemical leads) for drug development on related receptors.

In other words, the present invention also relates to a drug discovery method for identifying ligands, which bind to a first 7TM receptor and potentially bind to one or more further 7TM receptors, the method comprising the steps of i) to vii) as defined above and the further steps of

viii) selecting from one to about 100 further 7TM receptors which have the closest similarity scores to the first 7TM receptor,

ix) identifying ligands which potentially bind to those further 7TM receptors selected in step viii) by selecting ligands that bind to the first 7TM receptor.

In addition, the method described above may be combined with a screening step, to determine ligands, which do bind to a 7TM receptor. Therefore, the present invention additionally relates to a drug discovery method for identifying ligands which bind to a first 7TM receptor and to one or more further 7TM receptors, the method comprising the steps of i) to vii) as defined above and the further steps of:

viii) selecting from one to about 100 further 7TM receptors which have the closest similarity scores to the first 7TM receptor,

ix) screening ligands that bind to the first 7TM receptor against the selected 7TM receptors of step viii).

The process of transfer of a chemical starting point from one protein target to another related protein target is often referred to as chemogenomics. Thus, an efficient physicogenomic method to compare 7TM receptors having known ligands (known or potential drug molecules) with novel receptors lacking identified ligands allows for possibilities to identify lead structures for drug development since no previous information regarding ligands binding to the new receptor under investigation is needed. Furthermore, comparison of 7TM receptors having ligands with orphan receptors lacking identified endogenous ligands allows for the identification of lead structures for drug development also on orphan receptors since no previous information regarding ligands binding to the receptor under investigation is needed. Consequently, known ligands of closely related receptors could serve as good chemical starting points to identify lead structures against a receptor for which no agonist or antagonist are known.

In a further aspect, the present invention relates to a drug discovery method for identifying a potential lead compound for a first 7TM receptor, the method comprising the steps of i) to vii) as defined above and the further steps of

viii) selecting from one to about 100 further 7TM receptors which have the closest similarity scores to the first 7TM receptor,

ix) identifying ligands that bind to said one or more further 7TM receptors to construct a library including a potential lead compound for the first 7TM receptor.

The method for identifying a potential lead compound may additionally be linked to a screening step, so that libraries containing potential lead compounds may be narrowed down to give lead compounds. Hence, the present invention further relates to a drug discovery method for identifying a lead compound for a first 7TM receptor, the method comprising the steps of i) to vii) as defined in herein and the further steps of

viii) selecting from one to about 100 further 7TM receptors which have the closest similarity scores to the first 7TM receptor,

ix) identifying ligands that bind to said one or more further 7TM receptors to construct a library, and

x) screening said library against the first 7TM receptor to identify a lead compound for the first 7TM receptor.

Pharmacophore model

Having established physicogenomic relationships between 7TM receptors one can also derive pharmacophore models based on ligands, which are known, to bind to 7TM receptors related to the receptor under investigation. This allows for a more cost-effective process of retrieving and screening a limited number of compounds than under a conventional high-throughput screening (HTS) campaign usually conducted in larger pharmaceutical industries when looking for lead structures for a novel biological target.

Furthermore, a pharmacophore model that can be used for *in silico* screening or design of focused chemical libraries could be derived from analogously identified structures.

Hence, the present invention relates to a drug discovery method for constructing a pharmacophore model for a first 7TM receptor, the method comprising the steps of i) to vii) as defined in herein and the further steps of

viii) selecting from one to about 100 further 7TM receptors which have the closest similarity scores to the first 7TM receptor,

ix) identifying ligands that bind to said one or more further 7TM receptors to construct a pharmacophore model.

In one embodiment of the present invention the first 7TM receptor is one for which no ligands have been identified. Additionally, the first 7TM receptor may be an orphan receptor.

7TM receptors, which have similarity scores closest to each other, have most similar physicochemical properties of their binding sites. Therefore, when selecting 7TM receptors based on their similarity score, with the aim of choosing those with similar physicochemical properties of their binding sites, it is important to select those with the closest similarity scores. The number of further 7TM receptors which are selected in step vii) (above) may be from one to 50, from one to 25 or from one to 15.

Having defined the relevant binding site and assigned the proper physicochemical descriptors to selected amino acid residues involved in the binding site, the 7TM receptors are compared to each other by a suitable computerised mathematical model, which is capable of comparing a large number of receptors by an effective algorithm. Preferably, all identified 7TM receptors should be aligned and compared based on the physicochemical descriptors of pseudo-sequences derived from selected amino acid residues involved in the binding site. Accordingly, the present invention relates to any of the methods as described herein, wherein the method is executed by a computer under the control of a program and the computer includes a memory for storing said program.

Generic numbering system for 7TM receptors

The 7TM receptor superfamily is composed of many hundreds of receptors that may be further divided into smaller sub-families of receptors. The largest of these sub-families of 7TM receptors is composed of the rhodopsin-like receptors (also termed the family A receptors), which are named after the light-sensing molecule from our eye. The receptors are integral membrane proteins characterized by seven transmembrane (7TM) segments traversing the membrane in an antiparallel way, with the N-terminal on the extracellular side of the membrane and the C-terminal on the intracellular side. Within the membrane- embedded part and in some cases in the membrane proximal parts embedded in the aqueous environment surrounding the cell membrane, the polypeptide adopts a helical secondary structure. The lengths, and the beginning, centre and ends relative to the lipid bilayer membrane of these helices may be deduced from solved three-dimensional structures of the receptor proteins (Palczewski K. et al., *Science*, (2000) **289** (5480), 739-45). However, since the three-dimensional structure of only a single receptor has been solved to date, the helical lengths, and the beginning, centre and ends relative to the lipid bilayer membrane of each of the seven helices may be dissected by sequence analysis (J.M. Baldwin, *EMBO J.* (1993) **12**(4), 1693-703; J.M. Baldwin et al., *J. Mol Biol*, (1997) **272**(1), 144-64).

A useful tool in the analysis of 7TM receptors is the generic numbering system for residues of 7TM receptors. Within the many hundred members of the rhodopsin-like receptor family, a number of residues especially within each of the transmembrane segments are highly, but not totally, conserved - termed key residues. These residues may be used to direct an alignment of the primary protein sequences within the transmembrane segments together with other standard principles and techniques, well known to persons skilled in the art (for example hydrophobicity plots). Additionally a number of other residues occur within the transmembrane segments that are highly conserved, and these may be used to further direct an alignment of the transmembrane segments. These are particularly useful when a given key residue in a transmembrane segment has been substituted through evolution by another amino acid of a dissimilar physiochemical nature.

However, due to differences in the length of especially the N-terminal segment, the third intracellular loop 3 and the C-terminal segment, residues located at presumably structurally corresponding positions in different 7TM receptors are numbered differently in different receptors. However, based on the conserved key residues in each transmembrane segment, a generic numbering system has been suggested (JM Baldwin, *EMBO J.* (1993) **12**(4), 1693-1703; TW Schwartz, *Curr. Opin. Biotech.* (1994) **5,** 434-444). On the basis of the key residues present in the receptor family, the transmembrane segments are generically numbered. For example, in TM-II the highly conserved acidic function, aspartate (Asp) in the rhodopsin-like family is given the generic number 10, i.e. AspII:10, on the basis of its position in the helix. All other residues in the helix are hence numbered on this basis. In Figure 2.1 a schematic depiction of the secondary structure of a rhodopsin-like 7TM receptor is shown with one or two conserved, key residues highlighted in each transmembrane segment: AsnI:18; AspII:10; CysIII:01 and ArgIII:26; TrpIV:10; ProV:16; ProVI:15; ProVII:17.

In relation to the present invention, it is important that residues involved in, for example, ligand binding can be described in this generic numbering system. For example, in the β2-adrenergic receptor the main anchor point for the catecholamine agonists is a highly conserved aspartate 113 residue in TM-III (AspIII:08). Interestingly, the aspartate is located one helical turn deeper in the receptor structure to the counter-ion (GluIII:04) of the Schiff base in rhodopsin. Two serine residues at positions 204 and 207 in TM-V (SerV:09 and SerV:12) were identified as contact points for the hydroxy-functionality of

the catechol ring and were suggested also to be part of the control of the transition between the inactive and active state of the receptor. Moreover phenylalanine 290 and asparagine 293 on TM-VI (PheVI:17 and AsnVI:20 respectively) were identified as interacting with the catechol ring and the β-hydroxy of the agonists respectively. In catecholamine and 5-hydroxytryptamine receptors, residue VII:06 has been consistently identified as an interaction point for partial agonists and antagonists. Interestingly, a β2-selective non-catechol agonist TA-2005 was suggested to interact with tyrosine 308 on TM-VII (TyrVII:02), located one helical turn above VII:06, and was unaffected with respect to receptor activation by mutation of either serine residues 204 or 207 suggesting that different agonist activation modes may exist even within a particular receptor. Hence the agonist-binding site in the β2-adrenergic receptor is located between the transmembrane segments at a general position rather similar to the retinal binding site in opsin. Comparing the binding site of the β2-adrenergic receptor described above using the generic nomenclature to, for example, the dopamine D2-receptor, quickly identifies residues Asp114 in TM-III (AspIII:08), Ser194 and Ser197 in TM-V (SerV:09 and SerV:12), Phe390 and His393 (PheVI:17 and HisVI:20) hence suggesting that certain key residues in the binding of the catecholamines to the β2-adrenergic receptor are conserved to for example the dopamine receptor. Similarly the described binding site – or other residues suspected to be important in defining for example a binding site of a ligand - can easily be compared to even several and very distantly related receptors at the same time, where little or no conservation of the particular binding site of the endogenous catecholamine binding site exist, but where the corresponding residues nevertheless may be important for binding, for example, artificial non-peptide antagonists.

It is only in the rhodopsin-like receptor family that a generic numbering system has been established; however, it should be noted that although the sequence identity between the different families of 7TM receptors is very low, it is believed that they may share a more-or-less common seven helical bundle structure. Hence an analogous system may be developed for the other families of 7TM receptors, for example the family B class of receptors, composed of, among others, the glucagon receptor, the glucagon-like peptide (GLP) receptor-1, the corticotropin releasing factor (CRF) receptor-1, vasoactive intestinal peptide (VIP) receptor, pituitary adenylate cyclase-activating polypeptide (PACAP) receptor etc. (J.W. Tams et al., *Receptors Channels* (1998) **5**(2), 79-90). Again, on the basis of the key residues present in the family B class of receptors, the transmembrane segments are generically numbered (Figure

2.2). For example, in TM-I, the highly conserved hydroxy function, serine (Ser) is given the generic number 8, i.e. SerI:08 on the basis of its approximate position in the helix; in TM-II the highly conserved histidine (His) is given the generic number 6 i.e. HisII:06; in TM-III the highly conserved cysteine (Cys) is given the generic number 1 i.e. CysIII:01; in TM-IV the highly conserved proline (Pro) is given the generic number 13 i.e. ProIV:13; in TM-V the highly conserved asparagine (Asn) is given the generic number 14 i.e. AsnV:14; in TM-VI the highly conserved leucine (Leu) is given the generic number 9 i.e. LeuVI:09; in TM-VII the highly conserved glycine (Gly) is given the generic number 13 i.e. GlyVII:13. All other residues in the helices are hence numbered on this basis. Thus, all the techniques described in the present invention can be applied to the other families of 7TM receptors with minor modifications. This generic numbering system together with general knowledge of the 3D structure of the 7TM receptors and knowledge from systematic ligand binding site analysis makes it possible to predict or identify contact points for ligands based on the DNA sequence coding for the 7TM receptor (see Figure 2.1 and 2.2).

7TM receptors of interest in the present invention comprising 7 transmembrane domains include but are not restricted to G-protein coupled receptors, such as receptors for: acetylcholine, adenosine, norepinephrin and epinephrine, amylin, adrenomedullin, anaphylatoxin chemotactic factor, angiotensin, apelin, bombesin (neuromedin), bradykinin, calcitonin, calcitonin gene related peptide, conopressin, corticotropin releasing factor, , calcium, cannabinoid, CC-chemokines, CXC-chemokines, CX3C-chemokinees, cholecystokinin, corticotropin-releasing factor, dopamine, eicosanoids, endothelin, fMLP, $GABA_B$, galanin, gastrin, gastric inhibitory peptide, glucagon, glucagon-like peptide I and II, glutamate, glycoprotein hormone (e.g. FSH, LSH, TSH, LH), gonadotropin releasing hormone, growth hormone releasing hormone, growth hormone releasing peptide, Ghrelin, histamine, 5-hydroxytryptamine, leukotriene, lysophospholipid, melanocortins, melanin concentrating hormone, melatonin, motilin, neuropeptide Y, neurotensin, nociceptin, odor components , opiods, retinal, orexins, oxytocin, parathyroid hormone/parathyroid hormone-related peptides, pheromones, platelet-activating factor, prostanoids, secretin, somatostatins, tachykinins, thrombin and other proteases acting through 7TM receptor, thyrotropin-releasing hormone, pituitary adenylate activating peptide, vasopressin, vasoactive intestinal peptide and virally encoded receptors; and 7TM receptors coded for in the human genome and for which an endogenous receptor-ligand has or has not yet been assigned; *in particular:* adenosin, galanin, CC-chemokines, CXC-chemokines,

melanocortin, bombesin, cannabinoid, lysophospholipid, fMLP, neuropeptide Y, tachykinin, dopamine, histamine, 5-hydroxytryptamine, histamine, mas-proto-oncogene, melanin concentrating hormone receptor, Glucose-dependent insulinotropic polypeptide, Glucagon-like peptide-1 receptor, glucagon receptor, acetylcholine, oxytocin, human herpes virus encoded receptors, Epstein Barr virus induced receptors, cytomegalovirus encoded receptors and bradykinin receptors; and 7TM proteins coded for in the human genome but for which no endogenous receptor-ligand has yet been assigned; *preferably* the galanin receptor type 1, leukotriene B4 receptor, CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, CCR8, CCR9, CCR10, CXCR1, CXCR2, CXCR3, CXCR4, CXCR5, CXCR6, CX3CR1, melanocortin-1 receptor, melanocortin-3 receptor, melanocortin-4 receptor, melanocortin-5 receptor, MCH-1, MCH-2, GIP receptor, GLP-1 receptor, bombesin receptor subtype 3, cannabinoid receptor 1, cannabinoid receptor 2, EDG-2, EDG-4, FMLP-related receptor I, FMLP-related receptor II, NPY Y6 receptor, NPY Y5 receptor, NPY Y4 receptor, NK-1 receptor, NK-3 receptor, D1 receptor, D2 receptor (short), D2 receptor (long), D3 receptor, D4 receptor, D5 receptor, D6 receptor, D7 receptor, D7 receptor, D8 receptor, duffy antigen; US27, US28, UL33 and U78 from human cytomegalovirus; U12 and U51 from human herpes virus 6 or 7, ORF74 from human herpes virus 8, and histamine H1 receptor, MAS proto-oncogene, muscarinic M1 receptor, muscarinic M2 receptor, muscarinic M3 receptor, muscarinic M5 receptor, oxytocin receptor, XCR1 receptor, EBI2 receptor, RDC1 receptor, GPR12 receptor or GPR3 receptor, and 7TM receptors coded for in the human genome and for which an endogenous receptor-ligand has or has not yet been assigned. These 7TM receptors may be studied in a monomeric or in a dimeric form, which may be either homo-dimeric or heterodimeric.

Aligning 7TM receptor proteins

As appears from the above, it is important to align at least a part of the amino acid sequences of the receptors to be compared. In some cases this information may already be available and, therefore, such an alignment step may be omitted, but in other cases the method of the invention includes a step of alignment.

Firstly, sequence databases, such as SWISSPROT, SPTREMBL, EMBL, PIR etc. are searched for human GPCR sequences using the Sequence Retrieval System (network browser for databanks in molecular biology) SRS. The identified sequences are then aligned using conventional alignments algorithms such as ClustalW (Thompson J.D. Higgins D.G. & Gibson T. J. Nucleic Acids Research. (1994) 22, 4673-80) The

resulting alignment is manually inspected and refined if necessary, so that conserved generic sequence signatures within the seven transmembrane 7TM helices are satisfied (Palczewski. K. et al, Science, (2000) 289, 739-745).

Secondly, the helices of the 7TM receptor are identified based on hydrophobicity plots, the conserved residues within the sub-family of receptors and - for family A receptors - the sequence alignment to the recent published crystal structure of rhodopsin (OPSD).

Furthermore, the invention relates to methods as described herein, wherein step i) is included and the alignment is based on a model developed for 7TM receptors. The 7TM receptors of the present invention may be Class A, Class B, Class C or taste receptors

In one embodiment, the invention describes a method or methods described above, wherein step i) is included and the alignment is made with respect to transmembrane positioning of α-helices of 7TM receptors.

A method according to the invention involves the use of a pseudo-sequence. A pseudo-sequence is obtained from at the most 12 amino acid residues per 7TM helix, and at the most 12 amino acids in one or more extracellular loops, sequential or non-sequential, involved in one of more binding site. Furthermore, such a pseudo-sequence may comprise at the most 50 amino acid residues. In a specific embodiment at the most 8 such as, e.g., at the most 6 amino acid residues per 7TM helix or extracellular loop form the pseudo-sequence containing at the most 40 amino acid residues such as, e.g., at the most 30 amino acid redidues. In an embodiment of the invention only amino acid residues from at the most 6 such as, e.g., 5 helices are included in the pseudo-sequence.

A surprinsingly good reflection of the physicochemical environment in the binding site is conveyed by the relatively few amino acids invoked in the analysis of only a relatively short pseudo-sequence. This is supported by the fact that ligands binding to receptors that have been found to be related to the given receptor also could display affinity for the given receptor (cf. investigation of CRTH2 and identification of the related receptor angiotensin II and hence a angiotensin II ligand candesartan also binding to CRTH2).

A selection of relevant amino acid residues for the pseudo-sequence can be made by a person skilled in the art based on current knowledge on 7TM receptors as discussed in the paragraph "Generic numbering system for 7TM receptors". Examples of relevant literature are T. Klabunde, G. Hessler, Drug design strategies for targeting G-protein-coupled receptors. Chembiochem. 3 (2002) 928-44; D. R. Flower, Modelling G-protein-coupled receptors for drug design, Biochimica et Biophysica Acta 1422 (1999) 207-234; J. A. Bikker, S. Trumpp-Kallmeyer, and C. Humblet G-Protein Coupled Receptors: Models, Mutagenesis, and Drug Design Journal of Medicinal Chemistry 41 (1998) 2911-2927.

Although amino acid residues known to be important for small molecule ligand interactions of course are of interest in construction of the pseudo-sequence, the present invention is not limited thereto.

An example of such an alignment for a random set of selected 7TM receptor human sequences is shown in the table below. For this illustration, the sequences and identification codes for the 7TM receptors are retrieved from www.gpcr.org. The amino acid residues, in sequential or non-sequential order, are selected from different helices located in the binding site. For GPR44, the following amino acid residues, up to six per helix, from TM-III, TM-IV, TM-V, TM-VI and TM-VIII. have been selected. In an specific example given below, the selected residues for TM-VII correspond to VII-02 (Leu), VII-06 (Thr) and VII-09 (Ala) in the generic numbering. The rest are assigned analogously as described above.

TM Helix:	III	IV	V	VI	VII
GP44_HUMAN	HSFFMF	NTY	AKFA	WYHSEA	LTA

The amino acid residues, e.g. up to six per helix, in sequential or non-sequential order, are selected from III-04 to VII-09 to form the following 22 amino acid pseudo-sequences, which are used in the alignment and subsequent comparison.

GP44_HUMAN HSFFMFNTYAKFAWYHSEALTA
O2T1_HUMAN QHYLVGDGLSINFLFSLYAKVT
O7C2_HUMAN QIFIGCGSETEIFVLCLYSLVT
B3AR_HUMAN WTDVVTVSPVSSSWFFNRAFNG

PE24_HUMAN STLLSLTTTAASSSLVVNQDIA
APJ_HUMAN SSIFMYLAVGSTGWYHKYMFTS
O1E1_HUMAN QMFLGDHAHACFDVFLLYATMT
FML2_HUMAN VHIDLFLTNLHFGWYEGMAISA
ACTR_HUMAN IDFVLLTGMVVITWFVVMTFGI
5H4_HUMAN RTDVTTISPACSAWFFNDPWLG
NFF2_HUMAN SGQGVAIMSTVYRWLWMSDYHA
O2B2_HUMAN QLFLGSNSQHVDFLVTLYAKLG
AG2R_HUMAN ASVSLYASAGKNGWHQTDVMIA
O5U1_HUMAN QVFIASSGHKIHFRSARVFLVT
1019_HUM NLLSRTLNLHLYEFSIGSMFLT
C3X1_HUMAN TTFFFFVAQNTNGWYNIETLEA
5H6_HUMAN WTDVCSASPVASTWFFNQAFTG
BRB2_HUMAN VNISLYLSMNLNGWFQTDTTSA
O2F2_HUMAN QLSLGGNSQPTNIMFCLYIKVA
NMBR_HUMAN IPQLVGLAESIFYWNHYRSTRS
OXI2_HUMAN QMIHSMARISLSYYMISHRVNL
NTR2_HUMAN YYHEAYLAMINVSWYHRYCYNF
AG2S_HUMAN ASVSLYASAGKNGWHQTDVMIA
5H2A_HUMAN WIDVSTISIVGSSWFFNAVLVG
GP72_HUMAN SRQYLHFSHDTFLWLNVLSYHA
ACM1_HUMAN WLDYSNLWATTAAWYNVSTWYC
CKRA_HUMAN ISYSFHLAAAQVGQYSLDTLSA
TA2R_HUMAN MGMIGLLGPSLSLWLLITVLLA
OYD1_HUMAN QMVHYARRYGVAAYAFFHRINV
LGR5_HUMAN IGSISEKYSLLNCNVASSLKLV
O5V1_HUMAN QLFVVGNSHNINFWFLVYIRVS
FSHR_HUMAN AGTVSEAAPVCLDMISAASKVH
DADR_HUMAN WVDISTISPASSSWFFNLPFVG
GRPR_HUMAN IPQLVGLAESSFFWNHYRSSRA
AG22_HUMAN FGLTMFSSTAKNGWFHTDALIG
O2D2_HUMAN RLFLGCVSDSIAFLVFLYGKVA
O4F3_HUMAN QIIHGGHSQPLDYFPMYPHKIA
O2H3_HUMAN QIFLGTAWGQSTLVLSLYGKLA
HH1R_HUMAN WLDYSTWVIKTANWYFFIAHIG
CML1_HUMAN SNLIMFLSSTRFGWYHNELLTA

EBI2_HUMAN TAFYTYQTLLACGFYHIHMLVM
O2G1_HUMAN QMALGGSAYAIRLHSNRALNVT
ETBR_HUMAN VPQKVGLAEDLFYWLHRKLDIA
GPR1_HUMAN VFTVLDIGATKFGWYHSELITA
FML1_HUMAN IHVDLFLTVGRFGWFQAGTVSA
NK4R_HUMAN QNPIVFLAQHVIVWYHFTAYFA
GPR81_HUMA GLLARAGTLHMFEYSVRFLLLT
GPR6_HUMAN TVLVFALGPLRASWFACGSTLA

Table. Pseudo-sequence generic numbering alignment of selected human 7TM receptors.

Selection of binding site amino acid residues

The alignment of a pseudo-sequence mentioned above is used to identify binding sites or potential binding sites of the 7TM receptor. Such identification is necessary in order to enable designation of physicochemical descriptors to the amino acid residues involved in the (potential) binding site.

As outlined above, certain amino acids have been identified to be frequently involved in ligand interactions. Thus, amino acid residues facing the core of the 7TM bundle and the general 7TM ligand binding site defined by the transmembrane helices in addition to residue positions in the extracellular loops determined experimentally to be important for ligand binding are selected. Therefore, in an embodiment of the invention, the binding site includes amino acid residues located in one or more extracellular loops of the 7TM receptors. In a further embodiment of the invention, the binding site includes amino acid residues located in one or more subsites of the binding site and in one or more extracellular loops of the 7TM receptors.

Relevant examples on identification of residue positions of importance for ligand binding and recognition can be found in: Y Yamano, K. Ohyama, S. Chaki, D.F. Guo, T. Inagami, *Biochem. Biophys. Res. Commun.* **187** (1992) 1426-1431; S.A. Hjorth, H.T. Schambye, W.J. Greenlee, T.W. Schwartz, *J. Biol. Chem.* **269** (1994) 30953-30959; Y.H. Feng, K. Noda, Y. Saad, X.P. Liu, A. Husain, S.S. Karnik, *J. Biol. Chem.* **270** (1995) 12846-12850; K. Noda, Y. Saad, S.S. Karnik, *J. Biol. Chem.* **270** (1995) 28511-28514; H. Ji, M. Leung, Y. Zhang, K.J. Catt, K. Sandberg, *J. Biol. Chem.* **269** (1994) 16533-16536; Y. Yamano, K. Ohyama, M. Kikyo, T. Sano, Y. Nakagomi, Y. Inoue, N.

Nakamura, I. Morishima, D.F. Guo, T. Hamakubo et al. *J. Biol. Chem.* **270** (1995) 14024-14030; K. Noda, Y. Saad, A. Kinoshita, T.P. Boyle, R.M. Graham, A. Husain, S.S. Karnik, *J. Biol. Chem.* **270** (1995) 2284-2289; H.T. Schambye, S.A. Hjorth, J. Weinstock, T.W. Schwartz, *Mol. Pharmacol.* **47** (1995) 425-431); V. Nirula, W. Zheng, R. Sothinathan, K. Sandberg, *Br. J. Pharmacol.* **119** (1996) 1505-1507.

Physicochemical descriptors

Various physiochemical descriptors have previously been employed to classify and describe chemical features of peptides. In a method according to the present invention physicochemical descriptors are applied to amino acid residues located in or in the vicinity of the (potential) binding site. Use of such descriptors enables calculation of a similarity score between 7TM receptors so that a comparison of the individual 7TM receptors can easily be made.

Thus, structure-activity studies on peptides have shown the relevance of various physicochemical descriptors assigned to individual amino acid residues in describing biological properties by Quantitative Structure-Activity Relationships (QSAR), Principle Component Regression (PCR) and Partial Least-Squares (PLS) analysis. Such studies using various descriptors can be found in (among others): Simple parameterization of non-proteinogenic amino acids for QSAR of antibacterial peptides. Lejon, Tore; Svendsen, John S.; Haug, Bengt E. *Journal of Peptide Science* (2002) **8**, 302-306; Theory and applications of the integrated molecular transform and the normalized molecular moment structure descriptors: QSAR and QSPR paradigms. Molnar, Stephen P.; King, James W. *International Journal of Quantum Chemistry* (2001) **85**, 662-675; Modelling of the Amino Acid Side Chain Effects on Peptide Conformation. Sak, Katrin; Karelson, Mati; Jarv, Jaak. *Bioorganic Chemistry* (1999) **27**, 434-442; The application of the intermolecular force model to peptide and protein QSAR. Charton, Marvin. *Advances in Quantitative Structure-Property Relationships* (1999) **2**, 177-252; MS-WHIM Scores for Amino Acids: A New 3D-Description for Peptide QSAR and QSPR Studies. Zaliani, A.; Gancia, E., *Journal of Chemical Information and Computer Sciences* (1999) **39**, 525-533.; A validation study of molecular descriptors for the rational design of peptide libraries. Matter, H. *Journal of Peptide Research* (1998) **52**, 305-314; 3D-QSAR of angiotensin-converting enzyme inhibitors: functional group interaction energy descriptors for quantitative structure-activity relationships study of ACE inhibitors. Kim, Sanguk; Chi, Myung Whan; Yoon, Chang No; Sung, Ha-Chin. *Journal of Biochemistry and Molecular Biology* (1998) **31**, 459-467; Descriptors for

amino acids using MolSurf parametrization. Norinder, Ulf; Svensson, Peter. *Journal of Computational Chemistry* (1998) **19**, 51-59; Amino Acid Side Chain Descriptors for Quantitative Structure-Activity Relationship Studies of Peptide Analogs. Collantes, Elizabeth R.; Dunn, William J., III. *Journal of Medicinal Chemistry* (1995) **38**, 2705-13; Theoretical amino acid descriptors. Application to bradykinin potentiating peptides. Norinder, Ulf. *Peptides (New York,US)* (1991) **12**, 1223-7; Dedicated principal properties for peptide QSARS: theory and applications. Skagerberg, Bert; Sjöström, Michael; Wold, Svante. *Journal of Chemometrics* (1990) **4**, 241-53; Multivariate Parametrization of 55 Coded and Non-Coded Amino Acids. Jonsson, J.; Eriksson, L.; Hellberg, S.; Sjöström, M.; Wold, S. *Quant. Struct.-Act. Relat.* (1989) **8**, 204-209; Amino Acid Side Chain Parameters for Correlation Studies in biology and pharmacology. Fauchère, J.-L.; Charton, M.; Kier, L. B.; Verlooop, A.; Pliska, V. *Int. J. Pept. Protein Res.* (1988) **32**, 269-278; Peptide Quantitative Structure-Activity Relationships, a Multivariate Approach. Hellberg, Sven; Sjöström, Michael; Skagerberg, Bert; Wold, Svante. *Journal of Medicinal Chemistry* (1987) **30**, 1126-1135; Statistical Analysis of the Physical Properties of the 20 Naturally Occurring Amino Acids, Kidera, A.; Konishi, Y.; Oka, M.; Ooi, T.; Scheraga, J. A. *J. Protein Chem.* (1985) **4**, 23-55; Relations between Chemical Structure and Biological Activity in Peptides. Sneath, P. H. A. *J. Theor. Biol.*(1966) **12**, 157-195.

Such physicochemical descriptors applied to amino acids in peptides reflect the forces involved in ligand-receptor interactions and, accordingly, will reflect the interacting properties of the amino acid side chains in proteins, especially transmembrane receptors such as 7TM receptors.

For example, the amino acids may be described by surface volumes and log P of side-chains (Norinder, Ulf; Svensson, Peter. *Journal of Computational Chemistry* (1998) **19**, 51-59), ξ-angles and conformational strain energies ΔH_{strain} (Sak, Katrin; Karelson, Mati; Jarv, Jaak. *Bioorganic Chemistry* (1999) **27**, 434-442) or principle properties z (Hellberg, Sven; Sjöström, Michael; Skagerberg, Bert; Wold, Svante. *Journal of Medicinal Chemistry* (1987) **30**, 1126-1135) as shown in the table below.

		Surface	logP	angle chi	dH strain	Z1	Z2	Z3
		side chain	side chain					
Ala	A	37.988	-0.093	-77.85	0.00	0.07	-1.73	0.09
Arg	R	135.583	1.333	108.86	10.28	2.88	2.52	-3.44
Asn	N	72.969	-0.753	-55.42	6.17	3.22	1.45	0.84
Asp	D	68.631	-0.220	47.89	3.37	3.64	1.13	2.36
Cys	C	65.063	0.477	160.13	0.02	0.71	-0.97	4.13
Gln	Q	95.356	-0.344	134.68	1.42	2.18	0.53	-1.14
Glu	E	87.730	0.384	53.27	2.37	3.08	0.39	-0.07
Gly	G	7.206	-0.616	-148.03	n.d.	2.23	-5.36	0.30
His	H	100.603	0.249	24.57	3.38	2.41	1.74	1.11
Ile	I	98.084	1.529	-104.89	0.05	-4.44	-1.68	-1.03
Leu	L	102.422	1.678	-148.53	2.48	-4.19	-1.03	-0.98
Lys	K	116.134	1.092	47.61	2.78	2.84	1.41	-3.14
Met	M	107.039	1.581	6.37	2.79	-2.49	-0.27	-0.41
Phe	F	121.451	2.021	47.67	3.59	-4.92	1.30	0.45
Pro	P	77.446	1.005	169.73	10.49	-1.22	0.88	2.23
Ser	S	44.705	-0.733	30.24	0.73	1.96	-1.63	0.57
Thr	T	67.162	-0.043	46.04	3.71	0.92	-2.09	-1.40
Trp	W	153.493	2.379	178.69	0.08	-4.75	3.65	0.85
Tyr	Y	128.937	1.547	49.11	3.50	-1.39	2.32	0.01
Val	V	79.965	1.117	-106.54	6.54	-2.69	-2.53	-1.29

Ligands interact with biological target proteins *via* various forces such as ionic interactions, ion-dipole interactions, dipole-dipole interactions, hydrogen bond interactions, hydrophobic interactions, π-stacking interactions, edge-on aromatic interactions, cation-π interactions, dispersion and induction forces. Accordingly, physicochemical descriptors reflecting these interaction forces have successfully been employed in descriptors used in Quantitative Structure-Activity Relationships (QSAR), Principle Component Regression (PCR) and Partial Least-Squares (PLS) analysis of drug/ligand responses. "Quantitative structure-activity relationships and experimental design", U. Norinder and T. Högberg, "*Textbook of drug design and discovery*", Taylor and Francis, London, (2002), pp117-155.

The physicochemical descriptors can be experimentally derived and/or theoretically calculated. The descriptors can be seen to reflect hydrophobic properties, electronic properties, steric properties or hydrogen bonding capabilities. Some descriptors can be seen to reflect combinations of such properties, especially combinations of electronic and steric features. The present invention describes a method or methods wherein the physicochemical descriptors reflect 7TM receptor-ligand interaction features of the amino acid residues. Additionally, the physicochemical descriptors are chosen to reflect hydrophobic, electronic, steric, hydrogen bonding or other properties of the

amino acid residues. Yet further, the physicochemical descriptors may reflect 3-dimensional features of the amino acid residues.

The physicochemical descriptors of the present method may be selected from descriptors used in quantitative structure-activity relationships (QSAR), Principle Component Regression (PCR) and Partial Least-Squares (PLS) analysis of peptides.

Typical hydrophobic descriptors are e.g. Partition coefficient (logP), Calculated partition coefficient (clog P, Prolog P, Maclog P), Distribution coefficient (log D), Polar surface area, Nonpolar surface area, TLC retention time, HPLC retention time, and HPLC capacity factor (log k).

Typical steric parameters are e.g. Molecular weight (MW), van der Waals volume, van der Waals radius, Molar refractivity (MR), STERIMOL parameters (L, B_1, B_5), Total surface area, occupied volume by a residue buried in globular protein, and bulkiness defined as the ratio of the side-chain volume to its length.

Typical electronic parameters are e.g. Ionisation constant (pK_{COOH}, pK_{NH2}), Isoelectric point, Net charge at pH 7, ^1H NMR chemical shift, ^{13}C NMR chemical shift, Calculated interaction energies, Electronic Charge Index (ECI), Charge transfer for carbons (CT), Maximum electrostatic potential (V_{max}), Minimum electrostatic potential (V_{min}), Maximum local ionization energy (I_{max}), Minimum local ionization energy (I_{min}), Molecular Electrostatic Potential (MEP) on Connolly Molecular Surface, Energy of highest occupied molecular orbital (E_{HOMO}), Energy of lowest unoccupied molecular orbital (E_{LUMO}), Dipole moment (μ), Polarizability (α), Most positive partial charge on a hydrogen atom (qH+), Most negative partial charge in the molecule (q−), and Partial charges on the oxygen and carbon atoms (qC, qO) of the carbonyl group.

Thus, the physicochemical descriptors may be selected from molecular weight (MW), van der Waals volume, van der Waals radius, molar refractivity (MR), STERIMOL parameters (L, B_1, B_5), Parachor (P_r), polar surface area, non-polar surface area, total surface area, ionisation constant (pK_{COOH}, pK_{NH2}), isoelectric point, net charge at pH 7, partition coefficient (log P), calculated partition coefficient (clog P, Prolog P, Maclog P), distribution coefficient (log D), TLC retention time, HPLC retention time, HPLC capacity factor log k, ^1H NMR chemical shift, ^{13}C NMR chemical shift, steric and electrostatic 3D-property MS-WHIM indexes, calculated interaction energies, isotropic surface area

(ISA), electronic charge index (ECI), charge transfer for carbons (CT), Lewis basicity (LB), Lewis acidity (LA), maximum electrostatic potential (V_{max}), minimum electrostatic potential (V_{min}), maximum local ionization energy (I_{max}), minimum local ionization energy (I_{min}), conformational strain energy (ΔH_{strain}), molecular electrostatic potential (MEP) on Connolly molecular surface, local flexibility (Fr), flexibility index (Fb), chain flexibility (FO), occupied volume by a residue buried in globular protein, bulkiness defined as the ratio of the side-chain volume to its length, total energy (E_{total}), heat of formation (ΔH_f), energy of highest occupied molecular orbital (E_{HOMO}), energy of lowest unoccupied molecular orbital (E_{LUMO}), dipole moment (μ), polarizability (α), most positive partial charge on a hydrogen atom (qH+), most negative partial charge in the molecule (q−), partial charges on the oxygen and carbon atoms (qC, qO) of the carbonyl group, integrated molecular transform (FTm), integrated electronic transform (FTe), Integrated charge transform (FTc), normalized molecular moment (Mn), electronic moment (Me), charge moment (Mc), absolute electronegativity (EN), absolute hardness (HA). Such descriptors convey information on ligand-binding features in a biological target protein such as transmembrane receptors including 7TM receptors.

The physicochemical descriptors of amino acids or of amino acid side chains can also be obtained from principal component analysis (PCA) of the above-mentioned physicochemical descriptors, e.g. such as principal properties *z*-scales derived from collections of experimental data or with additional theoretical descriptors, MS-WHIM 3D-description matrices reflecting structural and electronic features of molecules, t-scores from interaction energies calculated with program GRID, and other combinations of descriptors mentioned above. C.f. Priolo et al., J. M. *Journal of Molecular Catalysis B: Enzymatic* (2001) **15**, 177-189, Lejon et al., *Journal of Peptide Science* (2001) **7**, 74-81, Zaliani et al., *Journal of Chemical Information and Computer Sciences* (1999) **39**, 525-533, Matter, H. *Journal of Peptide Research* (1998) **52**, 305-314, Sandberg et al, *Journal of Medicinal Chemistry* (1998) **41**, 2481-2491, Collantes and Dunn, *Journal of Medicinal Chemistry* (1995) **38**, 2705-13, Norinder, Ulf. *Peptides (New York, NY, United States)* (1991) **12**, 1223-7, Hellberg and Kem. *International Journal of Peptide & Protein Research* (1990) **36**, 440-4, Skagerberg et al. *Journal of Chemometrics* (1990) **4**, 241-53, Hellberg et al. *Journal of Medicinal Chemistry* (1987) **30**, 1126-1135. In other words, in the methods according to the present invention wherein step v) is included, a simplified measure of the physicochemical properties of the binding site is obtained from principal component analysis (PCA) of the physicochemical descriptors.

Each residue type may be assigned as many physicochemical descriptors as decided, providing additional details of chemical features of the binding site of interest. In the following, a bitmap for a given selection of binding site residues is denoted F. Other descriptors used in the references cited herein may be chosen or combinations of these or novel descriptors reflecting physicochemical properties of amino acids relevant for ligand receptor interactions may be selected.

The physicochemical descriptors according to the present invention may also include dummy parameters or indicator variables, e.g. 1 and 0. Said indicator variables may denote the absence or the presence of aromatic side chains, hydrophobic side chains, negatively charged side chains, positively charged side chains, polar side chains, hydrogen-bond donating side chains, hydrogen-bond accepting side chains and/or other selected features.

The binding site classification procedure using for example binary codes is illustrated in Figure 3.

In this example, a normalised string of bits, 0 or 1 representing chemical features of the binding site residues are generated. A set of five bits are assigned to each amino acid residue specifying the absence 0 or presence 1 of a certain chemical feature or characteristics. In this example the indicator variables correspond to the presence (TRUE = 1) or absence (FALSE = 0) of hydrophobic, aromatic, positively charged, negatively charged or polar features. For example, a tyrosine residue using such indicator variables will be represented by the bitmap fingerprint 1 1 0 0 1 (hydrophobic – aromatic – absent – absent - polar). The mapping process of physicochemical descriptors into a string containing information of all selected amino acids is usually carried out by conventional computerized methods. Certain chemical features may be considered more or less important than others, and weighted accordingly in the binding site classification. The present invention therefore describes a method wherein step v) is included and the physicochemical descriptors are weighted in step v).

An embodiment of the invention uses pseudo-sequences comprising at the most 50 amino acids obtained from at the most 12 amino acid residues per 7TM helix or extracellular loops, sequential or non-sequential, which are associated with physicochemical descriptors reflecting hydrophobic, electronic, steric, and hydrogen

bonding properties. A specific embodiment of the invention uses pseudo-sequences comprising at the most 40 amino acids obtained from at the most 8 amino acid residues per 7TM helix or extracellular loops, sequential or non-sequential, which are associated with physicochemical descriptors reflecting hydrophobic, electronic, steric, and hydrogen bonding properties. A preferred embodiment of the invention uses pseudo-sequences comprising at the most 30 amino acids obtained from at the most 6 amino acid residues per 7TM helix or extracellular loops, sequential or non-sequential, which are associated with theoretically derived physicochemical descriptors reflecting hydrophobic, electronic, steric, and hydrogen bonding properties.

Similarity Scores

It is desirable to quantify how similar a given receptor binding site or subsite is to other receptor binding sites and or subsites. Here we apply a number of different similarity measures to rank binding sites and their corresponding bitmaps. The measures are chosen due to their capabilities to handle large data sets and to iteratively, if needed, allow the investigation of relationships between combinations of different subsites or binding sites. The measures could handle different types of descriptors described herein and may be based upon a pattern recognition method, a Principal Component Analysis (PCA) reducing the number of descriptors to a few principal components, a Tanimoto Similarity Measure, a Tversky Similarity Measure or a Euclidian Distance Measure as described in Press, W.H; Flannery, B.P.; Teukolsky, S.A.; Vettering, W.T. Numerical recipes: The art of scientific computing; Cambridge University Press: 1986. The present invention relates to methods, wherein the generation of a similarity score in step v) is based upon a pattern recognition method. In an embodiment of the methods according to the present invention, the generation of the similarity score involves a Principal Component Analysis (PCA) reducing the number of descriptors to a few principal components.

The similarity measure applied are the Tanimoto Coefficient *TC*, Tversky similarity *TS*, and an Euclidian distance *d(F1,F2)* defined below.

Tanimoto Similarity Measure:

The Tanimoto coefficient between two bitmaps *F1* and *F2* is defined as

$$TC = BC / (B1 + B2 - BC)$$

Here *B1* and *B2* are the numbers of 1's in *F1* and *F2* respectively and *BC* is the number of 1's in common between *F1* and *F2*. *TC* is a value between 0 and 1. If *TC* = 1 *F1* and *F2* are identical. If *TC* = 0, F1 and F2 have no TRUE = 1 occasions in common. In a further embodiment of the methods according to the present invention the generation of the similarity score in step v) (above) is based upon a Tanimoto Similarity Measure: TC = BC / (B1 + B2 – BC).

Tversky Similarity Measure:

The Tversky coefficient between two bitmaps *F1* and *F2* is defined as

$$TS = BC / (\alpha * B1Unique + \beta * B2Unique + BC)$$

Here *B1Unique* and *B2Unique* are the number of unique 1's in *F1* and *F2* respectively. α and β are constants used to weight prototype and variant features. When $\alpha = \beta = 1$, this measure produces a symmetrical similarity metric identical to *TC*. In a still further embodiment of the methods according to the present invention, the generation of the similarity score in step v) is based upon a Tversky Similarity Measure: TC = BC / (α * B1Unique + β * B2Unique + BC), wherein α are prototype features and β variant features.

Euclidian Distance Measure

The Euclidian distance represents the geometric distance between the bitmaps *F1* and *F2*

$$d(F1,F2) = sqrt\ (F1-F2)^2$$

where *F1* and *F2* are vectors in a N dimensional space. In the situation where physicochemical descriptors are used, F1 and F2 are no longer bit strings but string containing physicochemical descriptors representing the binding site residues of interest. In yet another embodiment of the methods according to the present invention, the generation of the similarity score in step v) is based upon Euclidian Distance Measure: $d(F1,F2) = sqrt\ (F1 - F2)^2$.

Ranking of 7TM receptors

A ranking of the 7TM receptors based on the physicochemical properties of their binding sites may be obtained, which gives a good indication of the similarity between

them. In certain cases this is important, and in such cases, step vi) (above) is included.

The ranking with respect to the physicochemical properties assigned to the aligned pseudo-sequence is based upon similarity scores obtained according to the procedures described above, or from distances between coordinates in a Principle Component (PC) n-dimensional space, but it may also be based upon a 2- or 3-dimensional graphical representation. In the latter case, visualisation of the relationship and similarities between 7TM receptors is simplified.

Examples of receptors where sequence alignment and the physicogenomics approach give comparable relationships between 7TM receptors are typically illustrated by certain receptors with subclasses such as the neurokinin NK1 to NK4 receptors and muscarinic M1 to M5.

Thus, a model using theoretical descriptors will rank the receptors closest to the muscarinic M3 as M5 (3.4), M2 (3.7), M1 (3.9) and M4 (4.3), which is in accordance with findings that muscarinic antagonists are in principle fairly subtype-unselective. Likewise, the same model ranks the receptors closest to the neurokinin NK1 as NK3 (3.3), NK4 (3.3) and NK2 (3.6), followed by Adenosine A3R (3.6).

In contrast, the same model applied to histamine H2 will rank the closest receptors as adrenergic b1(3.6) and b3(3.7), whereas the closest histamine receptor is more remote, i.e. histamine H1 (4.3) and the remaining ones are even further away but very close to each other, i.e. H3 (5.5) and H4 (5.5).

The same model applied to GRP44 (CRTH2) discussed previously will rank the closest receptors as angiotensin AT2 (2.9), chemokine-like receptor 1 (3.1), bradykinin B2 (3.4), Chemokine Receptor type 10 (3.6), Chemokine Receptor 1 (3.6), and angiotensin AT1 (3.7), which where not identified in the phylogenetic tree as close neighbours.

In one embodiment, the amino acid residues, up to six per helix, are selected from TM-III, TM-IV, TM-V, TM-VI and TM-VIII to form the following pseudo-sequences, which are used in the alignment. The following rank order of the similarity of the receptors can be obtained by implying the given set of amino acids associated with theoretically derived physicochemical descriptors reflecting hydrophobic, electronic, steric, and hydrogen bonding properties:

Receptor:	Pseudosequence	Ranking
GP44_HUMAN	HSFFMFNTYAKFAWYHSEALTA	1
AG22_HUMAN	FGLTMFSSTAKNGWFHTDALIG	2
CML1_HUMAN	SNLIMFLSSTRFGWYHNELLTA	3
BRB2_HUMAN	VNISLYLSMNLNGWFQTDTTSA	4
CKRA_HUMAN	ISYSFHLAAAQVGQYSLDTLSA	5
C3X1_HUMAN	TTFFFFVAQNTNGWYNIETLEA	6
AG2R_HUMAN	ASVSLYASAGKNGWHQTDVMIA	7

The use of lead structures might be based on *in silico* searches of specific scaffolds identified, on a pharmacophore model derived from these compounds, on *in silico* searches of such pharmacophore model, on design and synthesis of chemical libraries encompassing specific scaffolds identified, on a pharmacophore model derived from these compounds to design and/or construct chemical libraries containing novel chemical features compatible with the pharmacophores, on a pharmacophore models derived from these compounds to specifically design and synthesise novel ligands or on other common technologies used in drug design. The present invention also relates to the use of a pharmacophore as described herein for *in silico* screening, for construction of a library or for design of a ligand.

Having compared and ranked the 7TM receptors to each other by a suitable computerised mathematical model based on the relevant binding site and associated physicochemical descriptors of amino acid residues occupying the binding site, one can also identify 7TM receptors with binding properties similar to a given receptor and use that information to facilitate the drug design process.

For example the methods of the present invention allow identification of receptors, which are likely to cause a selectivity problem during drug development of a drug interacting with a given receptor. These potentially interfering receptors could be subject to directed counter-screens, reducing the need to screen compounds very broadly on a large number of receptors in the drug discovery and development process. The present invention relates to the use of the methods described herein to identify receptors, which are likely to cause a selectivity problem during drug development of a drug interacting with a given receptor.

Analogously to the utilization of the method to identify similarities in binding sites, the same principle can be applied to identify differences in subsites of binding sites between 7TM receptors as means to improve receptor selectivity of a drug towards a given 7TM receptor. The information regarding where significant differences exist in subsites between related receptors can be used in the design of ligands with improved receptor selectivity of a drug towards a given 7TM receptor.

Legends to figures

Figure 1 illustrates the conventional phylogenetic analysis of the GPR44 (CRTH2) receptor,

Figure 2.1 shows a schematic depiction of the secondary structure of a rhodopsin-like 7TM receptor with one or two conserved, key residues highlighted in each transmembrane segment: AsnI:18; AspII:10; CysIII:01 and ArgIII:26; TrpIV:10; ProV:16; ProVI:15; ProVII:17,

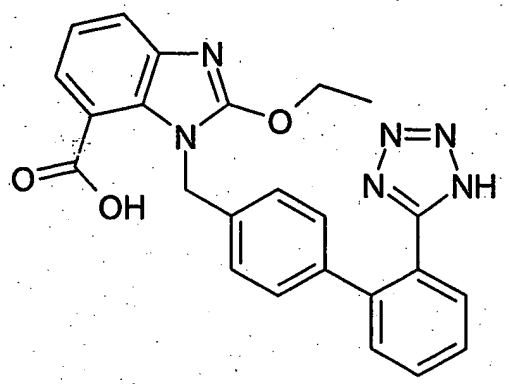
Figure 2.2 shows how the transmembrane segments are generically numbered based on of the key residues present in the family B class of receptors,

Figure 3 shows the binary 5-digit codes used to indicate absence or presence of physicochemical descriptors.

The following example is included for illustrative purposes and is not intended to limit the invention in any way.

Example

A model using theoretical size and electronic descriptors ranked the angiotensin AT2 and angiotensin AT1 receptors to be closely related to GRP44 (CRTH2) with respect to ligand-binding features in the binding site. Accordingly, among identified ligands, the known AT1 antagonist candesartan was found to inhibit [^{3}H]PGD2 of GRP44 with an IC_{50} of 2.1 μM.



CANDESARTAN

This example - describing the alignment procedure followed by amino acid selection and the other steps outlined above leading to the identification of AT1 as a GRP44-related receptor that serve as a source of discovering lead molecules for the new target GRP44 that can be used for in silico screening or design of focused chemical libraries - can analogously be applied to other receptors of interest.

Claims

1. A pseudo-sequence method for comparing a first 7TM receptor with one or more further 7TM receptors with respect to the physicochemical properties of selected amino acid residues of their binding sites, the method comprising the steps of:

i) optionally, aligning part of or all of the amino acid sequence of the first 7TM receptor with part of or all of the amino acid sequence of the one or more further 7TM receptors,

ii) selecting, in a sequential or non-sequential order, at the most 12 amino acid residues per helix and/or extracellular loops, which are involved in one or more binding sites of each 7TM receptor,

iii) forming a pseudo-sequence comprising at the most 50 amino acid residues from the selected sequential or non-sequential amino acid residues,

iv) for each 7TM receptor assigning one or more physicochemical descriptors to the amino acid residues of the selected amino acid pseudo-sequence involved in one or more binding sites,

v) optionally, for each 7TM receptor mathematically manipulating the physicochemical descriptors of step iv) to obtain a simplified measure of the physicochemical properties of the binding site,

vi) for each 7TM receptor generating a similarity score as defined herein by comparing the physicochemical descriptor or, if relevant, the simplified measure for the first 7TM receptor with the physicochemical descriptors or, if relevant, the simplified measures for the one or further 7TM receptors,

vii) optionally, ranking the 7TM receptors with respect to the physicochemical properties of their binding sites according to the similarity scores obtained in step vi).

2. A method according to claim 1, wherein the comparison is made without using data related to binding affinity of a ligand to a 7TM receptor.

3. A method according to claim 1 for classifying 7TM receptors according to the physicochemical properties of their binding sites.

4. A method according to claim 3, wherein the classification is made without using data related to binding affinity of a ligand to a 7TM receptor.

5. A method according to any of the preceding claims, wherein step ii) as defined in claim 1 comprises selecting, in a sequential or non-sequential order, at the most 11 such as, e.g., at the most 10, at the most 9, at the most 8, at the most 7 or at the most 6 amino acid residues per helix and/or extracellular loops, which are involved in one or more binding sites of each 7TM receptor.

6. A method according to any of the preceding claims, wherein step iii) as defined in claim 1 comprises forming a pseudo-sequence comprising at the most 50 such as, e.g., at the most 45, at the most 40, at the most 35 or at the most 30 amino acid residues from the selected sequential or non-sequential amino acid residues.

7. A drug discovery method for identifying ligands, which bind to a first 7TM receptor and potentially bind to one or more further 7TM receptors, the method comprising the steps of i) to vii) as defined in claim 1, 5 or 6 and the further steps of

viii) selecting from one to about 100 further 7TM receptors which have the closest similarity scores to the first 7TM receptor,

ix) identifying ligands which potentially bind to those further 7TM receptors selected in step vii) by selecting ligands that bind to the first 7TM receptor.

8. A drug discovery method for identifying ligands which bind to a first 7TM receptor and to one or more further 7TM receptors, the method comprising the steps of i) to vii) as defined in claim 1, 5 or 6 and the further steps of:

viii) selecting from one to about 100 further 7TM receptors which have the closest similarity scores to the first 7TM receptor,

ix) screening ligands that bind to the first 7TM receptor against the selected 7TM receptors of step viii).

9. A drug discovery method for identifying a potential lead compound for a first 7TM

receptor, the method comprising the steps of i) to vii) as defined in claim 1, 5 or 6 and the further steps of

viii) selecting from one to about 100 further 7TM receptors which have the closest similarity scores to the first 7TM receptor,

ix) identifying ligands that bind to said one or more further 7TM receptors to construct a library including a potential lead compound for the first 7TM receptor.

10. A drug discovery method for identifying a lead compound for a first 7TM receptor, the method comprising the steps of i) to vii) as defined in claim 1, 5 or 6 and the further steps of

viii) selecting from one to about 100 further 7TM receptors which have the closest similarity scores to the first 7TM receptor,

ix) identifying ligands that bind to said one or more further 7TM receptors to construct a library, and

x) screening said library against the first 7TM receptor to identify a lead compound for the first 7TM receptor.

11. A drug discovery method for constructing a pharmacophore model for a first 7TM receptor, the method comprising the steps of i) to vii) as defined in claim 1, 5 or 6 and the further steps of

viii) selecting from one to about 100 further 7TM receptors which have the closest similarity scores to the first 7TM receptor,

ix) identifying ligands that bind to said one or more further 7TM receptors to construct a pharmacophore model.

12. A drug discovery method according to claim 10, wherein the first 7TM receptor is one for which no ligands have been identified.

13. A drug discovery method according to claim 10 or 11, wherein the first 7TM receptor is an orphan receptor.

14. A method according to any of claims 7-12, wherein from one to 50 further 7TM receptors is/are selected in step viii).

15. A method according to any of claims 7-12, wherein from one to 25 further 7TM receptors is/are selected in step viii).

16. A method according to any of claims 7-12, wherein from one to 15 further 7TM receptors is/are selected in step viii).

17. A method according to any of the preceding claims, wherein the method is executed by a computer under the control of a program and the computer includes a memory for storing said program.

18. A method according to any of the preceding claims, wherein step i) is included and the alignment is based on a model developed for 7TM receptors.

19. A method according to claim 18, wherein the 7TM receptors are Class A, Class B, Class C or taste receptors.

20. A method according to any of the preceding claims, wherein step i) is included and the alignment is made with respect to transmembrane positioning of α-helices of 7TM receptors.

21. A method according to any of the preceding claims, wherein the binding site includes amino acid residues located in one or more extracellular loops of the 7TM receptors.

22. A method according to any of the preceding claims, wherein the binding site includes amino acid residues located in one or more subsites of the binding site and in one or more extracellular loops of the 7TM receptors.

23. A method according to any of the preceding claims, wherein the physicochemical descriptors reflect 7TM receptor-ligand interaction features of the amino acid residues.

24. A method according to any of the preceding claims, wherein the physicochemical descriptors are chosen to reflect hydrophobic, electronic, steric, hydrogen bonding or other properties of the amino acid residues.

25. A method according to any of the preceding claims, wherein the physicochemical descriptors reflect 3-dimensional features of the amino acid residues.

26. A method according to any of the preceding claims, wherein the physicochemical descriptors are selected from descriptors used in quantitative structure-activity relationships (QSAR), Principle Component Regression (PCR) and Partial Least-Squares (PLS) analysis of peptides.

27. A method according to any of claims 23-26, wherein the physicochemical descriptors are selected from molecular weight (MW), van der Waals volume, van der Waals radius, molar refractivity (MR), STERIMOL parameters (L, B_1, B_5), Parachor (P_r), polar surface area, non-polar surface area, total surface area, ionisation constant (pK_{COOH}, pK_{NH2}), isoelectric point, net charge at pH 7, partition coefficient (log P), calculated partition coefficient (clog P, Prolog P, Maclog P), distribution coefficient (log D), TLC retention time, HPLC retention time, HPLC capacity factor log k, ^{1}H NMR chemical shift, ^{13}C NMR chemical shift, steric and electrostatic 3D-property MS-WHIM indexes, calculated interaction energies, isotropic surface area (ISA), electronic charge index (ECI), charge transfer for carbons (CT), Lewis basicity (LB), Lewis acidity (LA), maximum electrostatic potential (V_{max}), minimum electrostatic potential (V_{min}), maximum local ionization energy (I_{max}), minimum local ionization energy (I_{min}), conformational strain energy (ΔH_{strain}), molecular electrostatic potential (MEP) on Connolly molecular surface, local flexibility (Fr), flexibility index (Fb), chain flexibility (FO), occupied volume by a residue buried in globular protein, bulkiness defined as the ratio of the side-chain volume to its length, total energy (E_{total}), heat of formation (ΔH_f), energy of highest occupied molecular orbital (E_{HOMO}), energy of lowest unoccupied molecular orbital (E_{LUMO}), dipole moment (μ), polarizability (α), most positive partial charge on a hydrogen atom (qH+), most negative partial charge in the molecule (q−), partial charges on the oxygen and carbon atoms (qC, qO) of the carbonyl group, integrated molecular transform (FTm), integrated electronic transform (FTe), Integrated charge transform (FTc), normalized molecular moment (Mn), electronic moment (Me), charge moment (Mc), absolute electronegativity (EN), absolute hardness (HA).

28. A method according to any of claims 17-25, wherein the physicochemical descriptors include indicator variables such as, e.g., 1 and 0.

29. A method according to claim 28, wherein the indicator variables denote absence or presence of aromatic side chains, hydrophobic side chains, negatively charged side chains, positively charged side chains, polar side chains, hydrogen-bond donating side chains, hydrogen-bond accepting side chains and/or other selected features.

30. A method according to any of the preceding claims including step v), wherein the physicochemical descriptors are weighted in step v).

31. A method according to any of the preceding claims including step v), wherein a simplified measure of the physicochemical properties of the binding site is obtained from principal component analysis (PCA) of the physicochemical descriptors.

32. A method according to any of the preceding claims, wherein the generation of a similarity score in step vi) is based upon a pattern recognition method.

33. A method according to any of the preceding claims, wherein the generation of the similarity score involves a Principal Component Analysis (PCA) reducing the number of descriptors to a few principal components.

34. A method according to any of the preceding claims, wherein the generation of the similarity score in step vi) is based upon Euclidian Distance Measure: $d(F1,F2) = \text{sqrt}(F1 - F2)^2$.

35. A method according to claim 28 or 29, wherein the generation of the similarity score in step v) is based upon a Tanimoto Similarity Measure: $TC = BC / (B1 + B2 - BC)$.

36. A method according to claim 28 or 29, wherein the generation of the similarity score in step v) is based upon a Tversky Similarity Measure: $TC = BC / (\alpha * B1Unique + \beta * B2Unique + BC)$, wherein α are prototype features and β variant features.

37. A method according to any of the preceding claims, wherein the step vii) is included.

38. A method according to any of the preceding claims, wherein the similarity score or, if relevant, the ranking is based upon a 2- or 3-dimensional graphical representation.

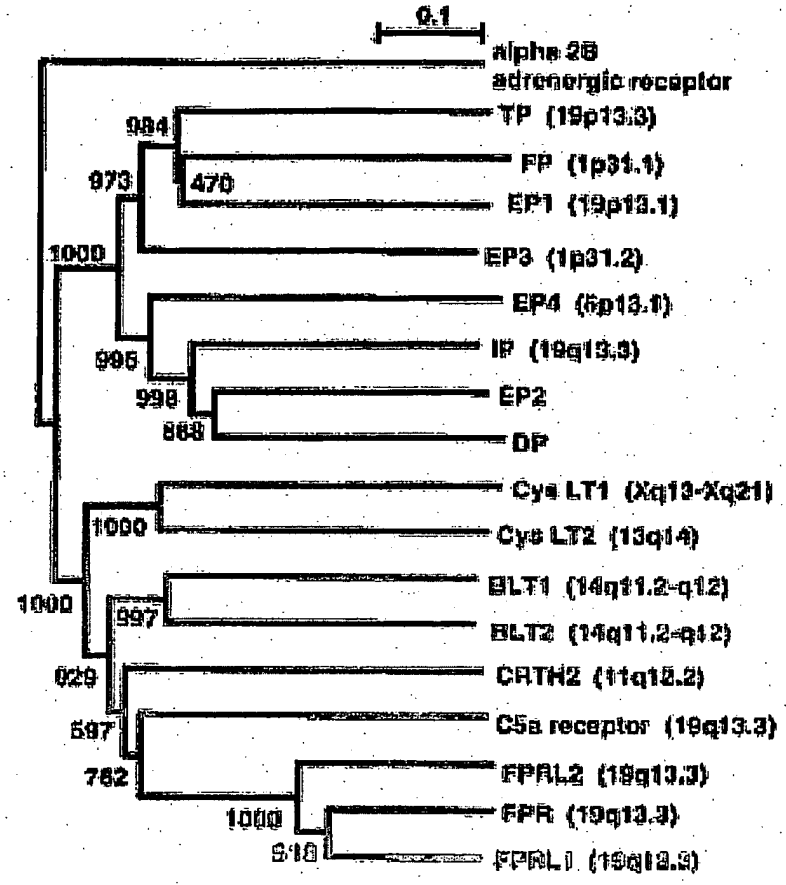
39. Use of a pharmacophore according to claim 11 for *in silico* screening.

40. Use of a pharmacophore according to claim 11 for construction of a library.

41. Use of a pharmacophore according to claim 11 for design of a ligand.

42. Use of a method according to any of claims 1-38 to identify receptors, which are likely to cause a selectivity problem during drug development of a drug interacting with a given receptor.

43. Use of a method according to any of claims 1-38 to identify differences in subsites of binding sites between 7TM receptors as means to improve receptor selectivity of a drug towards a given 7TM receptor.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FP (1p31.1)
EP1 (19p13.1)
EP3 (1p31.2)
EP4 (5p13.1)
IP (19q13.3)
EP2
DP
Cys LT1 (Xq13-Xq21)
Cys LT2 (13q14)
BLT1 (14q11.2-q12)
BLT2 (14q11.2-q12)
CRTH2 (11q12.2)
C5a receptor (19q13.3)
FPRL2 (19q13.3)
FPR (19q13.3)
FPRL1 (19q13.3)
984
973
470
1000
995
998
868
1000
1000
997
929
597
762
1000
918

Fig. 1

Family A

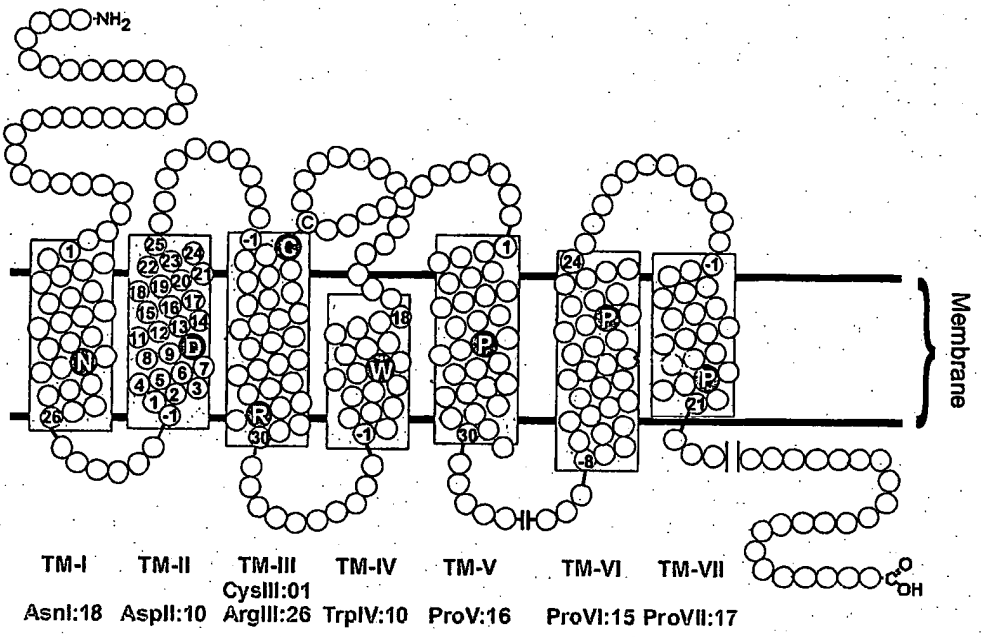

Fig. 2.1

Family B

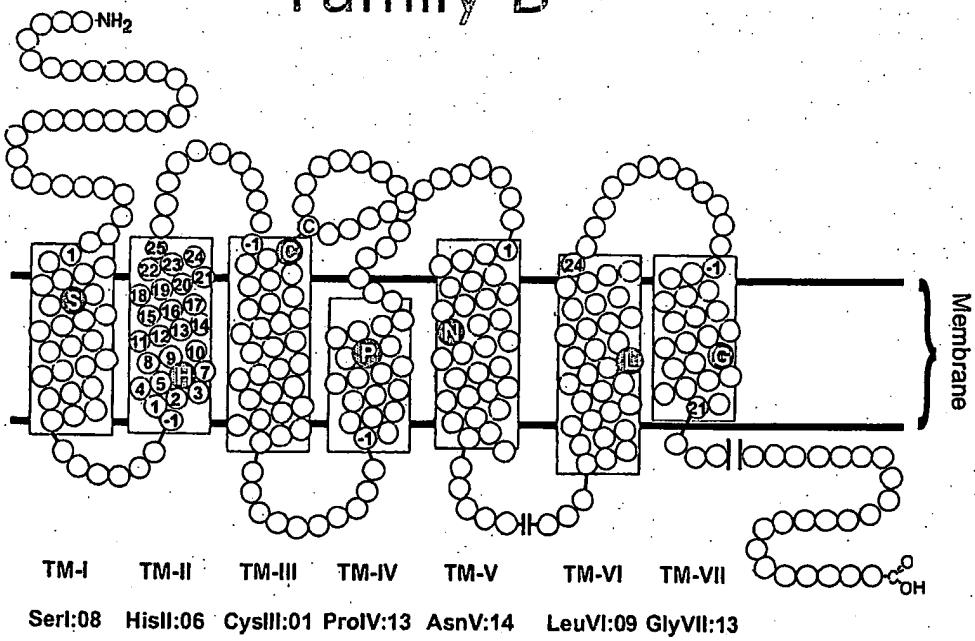

Fig. 2.2

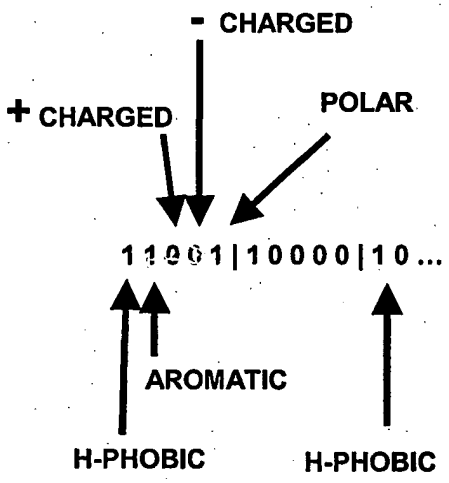
- CHARGED
+ CHARGED
POLAR
11001|10000|10...
AROMATIC
H-PHOBIC
H-PHOBIC

Fig. 3